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FRACTIONATION OF HUMAN PITUITARY GLANDS[1]

ALFRED E. WILHELMI

Abstract

A method of serial extraction of human pituitary glands is described. FSH, LH, and TSH are predominantly found in the first extract. A second extraction, under different conditions, yields principally growth hormone and some prolactin. A third extraction, under more extreme conditions of pH and temperature, yields, especially for acetone-preserved glands, an additional amount of growth hormone and the greater part of the prolactin. The method is presented as a contribution to the more efficient use of scarce and valuable human pituitaries.

Methods for the preparation of human growth hormone have been described by Li and Papkoff (1), by Raben (2), and by Lewis and Brink (3). A procedure for the isolation of human gonadotrophic hormones, modified from the method of Koenig and King (4), has been reported briefly by Steelman, Segaloff, and Mays (5). Growth hormone is also obtained by this method by applying Raben's procedure to the residue remaining after extraction of the gonadotrophins. Thus far, no general method has been described which makes possible the efficient separation, in good yield, of all or most of the major active principles of the human anterior pituitary gland. According to Li (6), the procedure of Li and Papkoff may be adapted to the isolation of other active principles, but the method does not seem to be highly efficient.

The aim of the work reported here has been to devise procedures for the extraction of all or most of the active principles of the human anterior pituitary gland in a form suitable for further purification, or for experimental use in man without further purification. Although an ideal solution of the general problem has not been attained, the procedure as it has been worked out thus far is presented (*a*) as a contribution to the more effective use of scarce and difficultly obtainable human pituitaries and (*b*) as a stimulus to further work. An account

[1]Manuscript received June 1, 1961.
Contribution from the Department of Biochemistry, Emory University, Atlanta 22, Georgia. This communication was originally made by Professor Wilhelmi on June 2, 1961, to the 4th Annual Meeting of the Canadian Federation of Biological Societies on the occasion of his election to Honorary Membership by the Canadian Physiological Society.

of some of the preliminary work on the development of this method has been presented elsewhere (7).

Source and Preparation of Materials

Human pituitaries, collected at autopsy and preserved under acetone, have been sent to this laboratory from a large number of hospitals collaborating in a program of collections organized under the auspices of the Endocrinology Study Section, National Institutes of Health. The glands are stored under acetone in a Deepfreeze at −20° C. When a sufficient number have been collected, the glands, still in the cold acetone, are minced in a Waring Blendor (three 30-second agitations) and the mince is freed of solvent by filtration through Whatman No. 1 paper on a Buchner funnel. After a wash with fresh cold acetone, the mince is briefly air-dried to remove most of the solvent and it is then dispersed in water, allowed to stand overnight in the cold, and then lyophilized. The dry material easily crumbles to a powder which is readily wetted by the aqueous solvents used in the extraction. The yield of dry powder is 80–120 mg per gland.

A few small batches of fresh frozen human pituitaries have also been received from individual collectors. These glands are prepared for extraction by mixing them with dry ice and grinding them to a fine powder in a dry food mill. When the dry ice has evaporated, the cold ground glands are dispersed in the extracting solvent.

Methods of Assay

All bio-assays were conducted with two doses of standard and of unknown, and the relative potencies were estimated from the parallel dose–response curves. Growth (GH) activity was measured by the 10-day gain in weight of 100 g hypophysectomized rats (8). The standard was the U.S.P. reference standard, with an activity of 1 unit per mg. Prolactin activity was measured by the increase in weight of the crop sac of 6-week-old White Carneau pigeons after four daily intramuscular injections of the substance under test (9). The standard was an ovine prolactin preparation (NIH-P-S3) containing 15 U.S.P. units per mg. Follicle-stimulating hormone (FSH) activity was measured by the augmentation method of Steelman and Pohley (10) using 25-day-old female Sprague–Dawley rats. The standard was an ovine FSH preparation (NIH-FSH-S1) containing 2.3 units per mg. (The unit in this case is the activity of 1 mg of the Armour FSH preparation 264-151-X.) Luteinizing hormone (LH) activity was estimated by the ovarian hyperemia method of Ellis (11), using immature female Sprague–Dawley rats. The standard was an ovine LH preparation (NIH-LH-S1), and the unit is defined as the activity of 1 mg of this preparation. Thyroid-stimulating hormone (TSH) activity was estimated by the method of Lamberg (12) using the P^{32} uptake of the thyroids of 2-day-old White Leghorn chicks. The standard was the U.S.P. reference standard. Adrenocorticotrophic hormone activity was not determined.

Extraction Procedure

A method of serial extraction is employed. The gland preparations are first extracted under conditions designed to bring into solution FSH, LH, and TSH. The residue is re-extracted under different conditions to obtain growth hormone and part of the prolactin. Finally, a third extraction, under somewhat more rigorous conditions of pH and temperature, is carried out to obtain the remainder of the prolactin, additional growth hormone, and the greater part of the ACTH.

For acetone-preserved material, the volume of solvent used is 20 ml per g, followed by a "wash" of 10 ml per g, in the first two extractions. For the third extract, the amount of solvent used is 16 ml per g, followed by a wash of 8 ml per g.

For fresh glands, the volume of solvent used was 4 ml per g, followed by a wash of 2 ml per g, in all three extractions.

All operations were carried out in a cold room at 3–5° C, unless otherwise indicated. Centrifuging was done in a Servall automatic refrigerated centrifuge at 0° C, using the SS-2 head at 6000 to 8000 r.p.m.

Extract A

The gland powder is suspended in 1.25 *M* ammonium sulphate solution, pH 4, and extracted for 4 hours. After centrifugation, the supernatant solution is decanted and the residue is resuspended in solvent and extracted for ½ hour. After centrifugation, the supernatant solution is added to the first extract and the volume of the combined extracts is measured. The residue is set aside in the cold for re-extraction (see extract B).

To the extract, at pH 4, add slowly solid ammonium sulphate (AS) to a final concentration of 3 *M* AS. After the precipitate has clearly flocculated and is settling (about 1 hour), centrifuge. Discard the supernatant solution. Take up the precipitate in water, neutralize, and dialyze until salt-free.

The dialyzed solution is transferred to a graduated cylinder and diluted to a protein concentration of about 1%. (Dilutions can be estimated on an expected yield of 40 mg per g of dry starting material at this stage.) The diluted solution is adjusted to pH 4, stirred for ½ hour, and centrifuged. The supernatant solution is collected in a graduated cylinder, and the insoluble residue is resuspended in one-fifth the original volume of water at pH 4 and stirred for ½ hour. The suspension is centrifuged, the supernatant solution is added to the first supernatant solution, and the total volume is recorded.

The precipitate is taken up in water, neutralized, dialyzed, and lyophilized: *fraction Aa*.

The combined supernatant solutions are adjusted to pH 7 and solid AS is added slowly to a final concentration of 2.2 *M* AS. When flocculation and settling have begun, centrifuge. Decant the supernatant solution and take up the precipitate in water, dialyze, and lyophilize: *fraction Ab*.

To the supernatant solution add slowly solid AS to a final concentration of

3 *M* AS. Let stand to flocculate and settle. Centrifuge. Discard the supernatant solution. Take up the precipitate in water, dialyze, and lyophilize: *fraction Ac.*

Extract B

Suspend the gland residue from extract A in water and adjust to pH 9. (From the volume of the wet residue, and on the assumption that it is essentially 1.25 *M* AS, it can be calculated that the concentration of AS will be about 0.15 *M*, when 20 ml of water per g of starting material is used in the second extraction.) Maintain the pH at 9 for the first few hours and extract overnight. Adjust the pH to 9, if necessary, and centrifuge. Decant the supernatant solution, recording its volume. Re-extract the residue with one-half the initial volume of water, pH 9, for 1 hour, and centrifuge. Decant the supernatant solution, recording its volume, and combine it with the first extract. For further treatment of the residue, see "Extract C" below.

To the combined extracts add slowly solid AS to a final concentration of 0.8 *M* AS and adjust to pH 7. Let stand to flocculate and settle. Centrifuge. Decant the supernatant solution. Take up the precipitate in water, dialyze, and lyophilize: *fraction Ba.*

To the solution at 0.8 *M* AS add solid AS slowly to a final concentration of 2.0 *M* AS. Let stand to flocculate and settle and then centrifuge. The supernatant solution is set aside, and the precipitate is treated as follows:

The expected dry weight of the fraction at this point is about 58 mg per g of starting material. Using this figure, calculate the volume of solvent that would be required to make a 1% solution of the precipitate. Then suspend the precipitate in one-half this volume of pH 5.1 phosphate buffer containing 0.45 *M* AS (0.052 *M* NaH_2PO_4; 0.0025 *M* Na_2HPO_4; 0.45 *M* $(NH_4)_2SO_4$) and record the total volume. The increase in volume, due to the volume of the precipitate, is regarded as essentially a solution of 2 *M* AS. Add to the system a volume of pH 5.1 phosphate buffer, *not* containing AS, calculated to adjust the volume of the precipitate to 0.45 *M* AS. Finally, dilute the system to 1% protein with pH 5.1, 0.45 *M* AS buffer. (The buffer is slightly diluted in this maneuver, but the final concentration of AS is kept at 0.45 *M*, and this is the important quantity.)

Extract for 1 hour and centrifuge. Decant the supernatant solution, suspend the precipitate in one-half the volume of pH 5.1, 0.45 *M* AS buffer, stir for 1 hour to disperse well, and centrifuge. Decant the supernatant solution, adding it to the first supernatant solution, and record the total volume. Take up the precipitate in water, neutralize, dialyze, and lyophilize: *fraction Bb-1.*

The combined supernatant solutions are taken to pH 4 and solid AS is added slowly to a final concentration of 1.25 *M* AS. Let stand to flocculate and settle, and then centrifuge. Decant the supernatant solution and set it aside (see below).

The precipitate is taken up in water, neutralized, and dialyzed. When the

system is salt-free it is transferred to a graduated cylinder and diluted with water to a concentration of about 1% protein, the final volume being based on an expected yield of 39 mg per g of starting material at this point. The solution is adjusted to pH 3.8, allowed to stir for 15 minutes, and then adjusted to the first point of definite flocculation (usually pH 4.3–4.4). Centrifuge. Decant the supernatant solution. Discard the precipitate. To the clear supernatant solution add slowly solid AS to a final concentration of 1.25 *M* AS, and adjust to pH 4.0. When flocculation and settling are clearly under way, centrifuge. Decant the supernatant solution. Take up the precipitate in water, neutralize, dialyze, and lyophilize: *fraction Bb-2*.

The supernatant solution is treated with solid AS to a final concentration of 3 *M* AS. Centrifuge. Discard the supernatant solution. Take up the precipitate in water, neutralize, dialyze, and lyophilize: *fraction Bb-3*.

The supernatant solution at pH 7, 2 *M* AS, is treated with solid AS to a final concentration of 3 *M* AS and centrifuged. Discard the supernatant solution. Take up the precipitate in water, dialyze, and lyophilize: *fraction Bc*.

Extract C

The gland residue from extract B is suspended in 0.1 *N* HCl at room temperature and extracted overnight. Centrifuge. Decant the supernatant solution and suspend the residue in one-half the volume of 0.1 *N* HCl, extract it for 1 hour, and centrifuge. Add the second supernatant solution to the first one and record the total volume. Discard the gland residue.

The initial pH of the extract is about 1.5. Adjust the pH to 3.0, add solid NaCl to 12% saturation (about 0.7 *M*), and transfer to the cold room to chill. When the solution has cooled, it is centrifuged. Decant the supernatant solution. Take up the precipitate in water, neutralize, dialyze, and lyophilize: *fraction Ca*.

The supernatant solution is saturated with NaCl in the cold. Let stand overnight. Centrifuge. Discard the supernatant solution. Take up the precipitate in water, neutralize, dialyze, and lyophilize: *fraction Cb*.

The procedure outlined above applies to acetone-preserved glands. The same process can be applied to fresh glands, with the following changes. (1) The 3 *M* AS precipitate of the A extract is neutralized, dialyzed, and lyophilized. This fraction is then extracted with water, at pH 4, in a 1% suspension. (2) The 2 *M* AS precipitate of the B extract is dialyzed and lyophilized. This fraction is then extracted, in 1% suspension, with the pH 5.1, 0.45 *M* AS buffer. (3) The pH 4, 1.25 *M* AS precipitate from this extract is dialyzed and lyophilized. The fraction is then redissolved in water, at pH 3.8, 1% protein.

Results

At the present writing, 587 g of powder from acetone-preserved glands, in seven lots, varying from 58 to 125 g, has been worked up by this procedure. Four smaller lots of fresh glands have also been worked up. Table I presents

TABLE I

Yields of fractions obtained from seven experiments with a total of 587 g of powder from acetone-preserved glands, and 83 g of fresh glands

	Acetone-preserved (587 g dry)			Fresh (83 g wet)	
Fraction	g	mg/g	mg/g (wet)*	g	mg/g (wet)
Aa	12.22	20.8	3.47	0.537	6.6
Ab	3.01	5.1	0.85	0.518	6.3
Ac	5.21	8.9	1.48	0.638	7.8
		34.8	5.80		20.7
Ba	12.47	21.2	3.53	1.083	13.2
Bb-1	7.01	11.9	1.98	0.769	9.4
Bb-2	10.13	17.3	2.88	1.385	17.0
Bb-3	2.90	5.0	0.83	0.181	2.2
Bc	9.06	15.3	2.55	0.285	3.2
		70.7	11.77		45.0
Ca†	36.45	68.6	11.43	0.324	3.9
Cb†	12.79	24.1	4.00	0.158	1.9
		92.7	15.43		5.8

*Calculated, factor 6, on basis of estimated 16% dry material derived from acetone treatment of fresh glands.
†Based on a total of 531 g of dry powder (the C extract from the seventh experiment was made by a different procedure).

the total weights and yields per gram of starting material of the fractions obtained from the acetone-preserved glands, and, for comparison, the data from a single run on 83 g of fresh glands. The yields of fractions in successive runs are fairly uniform, varying mainly with how cleanly prepared each batch of glands has been. It will be observed that fresh glands yield more material in extracts A and B, far less material in extract C. With acetone-preserved glands, the final acid extract is essential to the recovery of good yields of growth and prolactin activity; this is less important with fresh glands, in which these activities are mainly found in the B extract. It was noted in preliminary work (7) that although the yields of material in the A extract were much higher with fresh glands, the total gonadotrophic activity extracted was much the same as that extractable from acetone-preserved glands. The latter material therefore yields fractions of higher activity, and is advantageous for the gonadotrophins. This advantage is outweighed, however, by the higher yields of growth and prolactin activity in the B extract from fresh glands, as will be seen below.

Thorough bio-assay of all 10 fractions obtained in this procedure for all of the active principles has not yet been carried out. The picture is most complete for growth hormone activity, upon which our interest has been concentrated. Table II presents data for both acetone-preserved and fresh glands. It was expected that at pH 4 and 1.25 *M* ammonium sulphate little or no growth hormone activity would be extractable. This is corroborated by the observation on the 3 *M* ammonium sulphate precipitate from the A extract cited in Table II. In the B extract it will be seen that most of the growth activity is concentrated in fraction Bb-2, and that the specific activity is already high.

TABLE II

Distribution of growth activity in extracts A, B, and C

Fraction	Acetone-preserved			Fresh	
	units/mg	units/g	units/g wet	units/mg	units/g wet
A (3 *M* AS ppt.)	—	—	—	0.03	0.6
Ba	0.2	4.2	0.7	0.4	5.3
Bb-1	0.15	1.8	0.3	0.4	3.8
Bb-2	0.8	13.8	2.3	1.0	17.0
Bb-3	0.05	0.25	0.04	0.3	0.7
Bc	—	—	—	—	—
Ca	0.2	13.7	2.3	0.23	0.9
Cb	—	—	—	—	—

It will be noted that, with acetone-preserved material, as much growth activity is found in fraction Ca as in fraction Bb-2. The advantage of using fresh glands for the isolation of growth hormone is obvious: a much higher total yield of activity is obtained, and this is advantageously concentrated in fraction Bb-2.

The distribution of prolactin activity closely follows that of growth hormone. It was not expected that significant prolactin activity would be found in the A extract, but this point has not yet been tested by bio-assay. The observations have been concentrated on the B and C extracts, and these are summarized in Table III. From data presented by Li (6) it was expected that, in the preparation of fraction Bb-1 by extraction of the pH 7, 2 *M* AS precipitate of the

TABLE III

Distribution of prolactin activity in extracts B and C

Fraction	Acetone-preserved			Fresh	
	units/mg	units/g	units/g wet	units/mg	units/g wet
Bb-1	2.2	26.2	4.4	1.2	11.3
Bb-2	2.0	34.6	5.8	2.8	47.6
Ca	2.5	171.5	28.6	0.2	0.8

B extract with the pH 5.1, 0.45 *M* AS buffer recommended by Li and Papkoff (1), most of the prolactin activity would remain behind. For both acetone-preserved and fresh glands, in our procedure, this is not the case. Most of the prolactin activity accompanies the growth hormone into solution and is found in fraction Bb-2. With acetone-preserved glands, the final acid extract at room temperature brings out nearly three-fourths of the total prolactin activity found, whereas the additional yield from fresh glands is negligible. Acetone-preserved glands still yield 60–70% of the prolactin activity found in fresh material. It is of interest to note that the total prolactin activity found in fresh glands in these experiments agrees very well with the estimates made in 1939 by Chance, Rowlands, and Young (13) on alkaline extracts of fresh whole human pituitaries.

If the Ca fraction from acetone-preserved glands is extracted with the pH 5.1, 0.45 *M* buffer, most of the growth and prolactin activities go into solution together, and are precipitated again together at pH 4 and 1.25 *M* AS. The specific activities of this precipitate are 1 unit per mg for growth hormone and 7–9 units per mg for prolactin. Other attempts at separation of the two activities have not so far been successful. It would appear, however, from the widely different ratios of the two activities in different fractions, that they can be separated, as has been done with these active principles in other species.

In the preliminary work on this method (7) it was observed that over 90% of the total FSH activity was obtained in simple aqueous acid extracts of either acetone-preserved or fresh glands. Since FSH is soluble at pH 4 and 1.25 *M* AS, it was expected that most of this activity should be found in the A extract. Some support for this expectation is found in the observation that fraction Bc, in which any FSH not extracted initially should be found, had no FSH activity at doses of 150 and 450 μg. The specific activity of this fraction cannot be greater than 0.1 unit per mg. Further support for this expectation is found in the observation that FSH activity is highly concentrated in the fraction Ac. In Table IV data are presented on the yields of FSH in this fraction in four

TABLE IV

Yields of FSH in fraction Ac

Expt.	Acetone powder (g)	Fraction Ac (mg)	FSH activity	
			units/mg	units/g
1	85	910	6.0	64
2	67	713	13.0	138
3	80	867	11.0	119
4	126	463	20.0	74
5	258	2427	4.0	37

individual runs as well as on a fifth preparation made from a pool of the "A" precipitates (pH 4, 3 *M* AS) from three of the early experiments during which the steps of the procedure were being worked out. It will be noted that some variation in the yield and in the specific activity of the fraction is encountered, but that when the yield is low, the specific activity is high, so that the activity per unit weight of starting material is somewhat more uniform. Experiment 5 is an exception to this, but the fraction was involved in many more steps in its preparation, and it is not truly representative of the procedure in its present form as described. The average yield over all the experiments done so far is 84 units per g of dry powder, and this corresponds very closely to the yields obtained in whole extracts of small lots of glands during the trials of the procedure. For this reason it is not expected that any considerable amounts of FSH activity will be found in other fractions. A systematic study of this point is being planned.

The least satisfactory aspects of the method are the recoveries of TSH and LH. In preliminary studies (7) it was found that an extract made at pH 4, in

0.25 *M* AS, appeared to yield a high proportion of both of these activities; a second extract, made at pH 7.5 and 0.25 *M* AS, drew out only 6% of the activities present in the first extract. Furthermore, the yields were of such an order (3000 units per kg fresh weight) as to suggest that nearly all the activity present in the glands was being extracted. The expectations aroused by these observations are not realized in the present work. Fraction Ab, in which LH and TSH should be found together, has varied, in successive assays from 0.1 to 0.3 unit of TSH per mg, and from 0.4 to 0.6 unit of LH per mg. A glance at Table I will show that the recoveries of activity in fraction Ab, per unit weight of starting material, are only a fraction of what they should be. We have not yet had time to explore thoroughly the distribution of LH in all the fractions. For TSH, it can be said that fractions Aa and Ca contain no activity, but that a small amount of activity is found in fraction Bb-3, where it would appear if any additional activity were extracted in the B extract. The amount of TSH found, however, is no more than was found in the entire B extract in the preliminary trials mentioned above. It has also been observed that some fractions, assayed immediately after preparation, have exhibited satisfactory TSH activity, but that only about 25% of the activity could be found in these fractions when they were assayed again some weeks or months later. The TSH activity of human pituitaries may therefore not be stable, and special precautions may be required to preserve it during extraction and fractionation. For both LH and TSH, therefore, further work must be done.

The distribution of ACTH has not been studied systematically in these experiments. Preliminary work (7) seemed to show that only minor amounts of ACTH are found in the first two extracts. Fraction Cb, in which by far the greater part of the ACTH is expected, has been "assayed" by an as yet rough and unconventional method (14), involving the comparison of its effect on the output of free fatty acids by the rat epididymal fat pad with the effect of a highly purified ACTH preparation (80 U.S.P. units/mg). The estimate for the pooled Cb fraction is about 1 unit per mg, 24 units per g of acetone powder, or 4 units per g fresh weight, which agrees with earlier estimates made on whole extracts.

Discussion

From the data and the descriptions presented above it is obvious that the present method of fractionating human pituitaries still falls considerably short of an ideal procedure, and that further work must be done to define the current procedure more completely and to improve it. The method does yield satisfactory amounts of growth hormone, prolactin, and FSH, in forms readily susceptible of further purification or even useful without further purification for studies in human subjects. Although the yields of LH are disappointingly low at present, the specific activity of the fraction containing this hormone is high and can easily be improved. The recovery of TSH seems to involve special problems of stability.

None of the growth hormone preparations made so far have been completely free of prolactin activity, and in the course of partial purification of the prolactin fractions, none of these has been free of growth activity. The properties of these two hormones in human pituitary extracts are closely similar, and the problem of their separation is the most important one to be solved in the next stage of development of the method.

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BLOOD AMINO ACID STUDIES

I. A MICROMETHOD FOR THE ESTIMATION OF FREE LYSINE, METHIONINE, AND THREONINE[1]

J. M. McLaughlan, F. Noel, A. B. Morrison, and J. A. Campbell

Abstract

By a modification of conventional methods of microbiological assay it was possible to determine the free lysine, methionine, and threonine in 0.2 ml of rat plasma. The small amounts of lactic acid produced in the microassay were estimated photometrically by measurement of the color change of bromthymol blue. Repeated analysis of samples on different days indicated no significant effect of days and the confidence limits for the assays were approximately ±6% ($p = 0.05$). Mixtures of foods containing bread and various supplementary proteins were fed to young male rats for 24 hours and then plasma free lysine and methionine were measured. There was a direct relation between plasma free lysine levels and the lysine content of the diet. The method permits the accurate estimation of amino acids in blood of individual rats or in small samples of human plasma.

It has been known for several years that, in general, plasma levels of certain free amino acids reflect the pattern of those amino acids in the food ingested. Recent studies with chicks (1, 2), dogs (3), and rats (4) have demonstrated that measurement of plasma free amino acids after a meal of a test protein may yield useful information concerning the nutritive value of the protein. One of the difficulties which has retarded studies on plasma amino acids has been the large volume of plasma required for analytical purposes. Micromethods of amino acid assay, depending upon scaling down the volume of culture medium (5, 6) do not seem to be widely used and investigators have usually pooled blood from several animals when working with chicks (1, 2) and rats (5).

A study of possible micromethods led to the proposed procedure by use of which it is possible to measure the free lysine, methionine, and threonine in 0.2 ml of rat plasma. It is the purpose of this communication to describe the method for lysine in detail and to demonstrate its usefulness in determining the level of free lysine in the plasma of individual rats given different mixed diets. Directions pertinent to the microassay of methionine and threonine are also given.

Method

Preparation of Protein-free Plasma

This is a modification of the method of Hier and Bergeim (7). Anesthetize the rat by dropping it into a jar containing ether for approximately 10 seconds and then decapitate the animal with sharp scissors collecting the blood in heparinized tubes. Centrifuge the blood at 1500 r.p.m. for 5 minutes and

[1]Manuscript received June 15, 1961.
Contribution from the Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Canada.

remove the plasma within 1 hour of collection. Store the plasma at −20° C until amino acids are assayed.

Measure 1.28 ml of plasma into rimless test tubes (15×150 mm) containing 1.8 ml of distilled water and shake the tubes for several seconds. Add 0.45 ml of 0.6 *N* H_2SO_4 and shake the mixture for several seconds. Add 0.32 ml of a 10% solution of sodium tungstate and shake the mixture vigorously for 2 minutes. Centrifuge the tube for 5 minutes at 3000 r.p.m. and then decant the protein-free plasma. Adjust the pH of the solution to 6.8 with 0.1 *N* NaOH. As in the original method (7) a dilution of approximately 1 to 3 occurs during the preparation of the protein-free plasma.

Microbiological Assay for Lysine

Prepare a standard stock solution of L-lysine hydrochloride to contain 20 μg/ml of L-lysine; make a dilute standard working solution, fresh daily, containing 1 μg of L-lysine per ml. Dilute samples to contain approximately 1.0 μg/ml of lysine. Prepare a series of tubes (13×100 mm) in triplicate to contain 0.0, 0.1, 0.2, 0.3, 0.4, and 0.5 ml of the standard working solution of lysine. Add water to each tube to make 0.5 ml. Prepare a series of tubes in duplicate (triplicate if there is sufficient sample) to contain 0.2 and 0.4 ml of the sample. Add water to each tube to make 0.5 ml. Randomize the tubes throughout the racks, cover to minimize microbial contamination, and autoclave at 15-lb pressure for 5 minutes. Autoclave the basal medium at the same time but in a separate covered container. The basal medium is prepared at one third of the strength recommended in the original method (8).

To the cooled basal medium add the washed inoculum of *Leuconostoc mesenteroides* P-60 at the rate of 10–12 drops (density approximately 40 on the Coleman model 11) per 100 ml of basal medium. Swirl the medium to mix the inoculum and then add 0.5 ml of medium aseptically to each tube. (An automatic-filling hand syringe is very convenient for this purpose.) Incubate cultures at 37° C for 20–40 hours.

After incubation add 5 ml of an aqueous 0.004% solution of bromthymol blue (BTB) to each tube. Adjust the wavelength on the Coleman model 11 spectrophotometer, or other suitable instrument, to 610 mμ and set the instrument at 85% transmission with the pooled cultures containing the largest dosage level of lysine. Read the transmittance of each culture; plot the standard response curve and estimate the potency of each sample by interpolation from the standard curve.

Experimental and Results

A typical growth–response curve for L-lysine is given in Fig. 1. The total range in percentage transmittance was usually about 45 units. It is evident from Fig. 1 that solutions containing as little as 0.2 μg/ml of lysine may be estimated readily.

The reproducibility of the method was tested by assaying 12 samples on each of 3 days. Twelve different samples of protein-free rat plasma were chosen

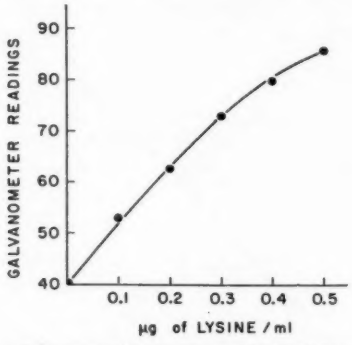

FIG. 1. Response of *L. mesenteroides* P-60 to L-lysine.

having a range of lysine concentrations of 20 to 40 µg/ml. Data for the repeated assays are given in Table I. Analyses of variance were carried out to determine the error of the assay; the mean square for residual error indicated that the limits of error for individual assays were about $\pm 6\%$ ($p = 0.05$). The effect of days on sample values was not significant.

TABLE I

Reproducibility of the microassay for lysine

Sample	Lysine found (µg/ml)		
	Day 1	Day 2	Day 3
A	39	39	36
B	36	36	35
C	39	36	37
D	41	40	40
E	31	28	30
F	30	29	28
G	31	32	30
H	33	35	33
I	26	25	25
J	28	27	25
K	20	20	22
L	23	23	23

The microassay for lysine was then compared with the conventional lysine assay of Steele *et al.* (8) scaled down to a total volume of 0.5 ml. Single doses of 30 individual samples of rat plasma were assayed by both methods. A comparison of values found by the two methods is given graphically in Fig. 2. Except for one sample, there was good agreement between the results by the two procedures indicating that the proposed micromethod gave essentially the same results as the conventional microbiological assay.

The essential features and validity of the micromethod have been illustrated with the assay for lysine but similar studies have also established the validity and reliability of the microassay for methionine. The conditions for the micro-

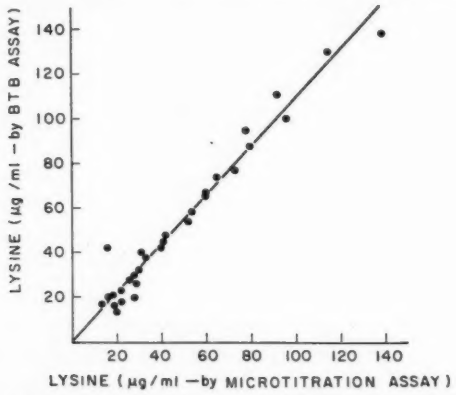

FIG. 2. Comparison of plasma free lysine values for 30 samples of rat plasma by conventional microtitration assay and by the proposed bromthymol blue (BTB) method.

assay of threonine using *Streptococcus faecalis* 9790 have also been determined and instructions pertinent to lysine, methionine, and threonine assays are given in Table II. Although the size of the inoculum is not critical it is probably advisable to use a relatively light inoculum in order to keep acid production low in the blank tubes.

TABLE II

Data pertinent to the microassay of lysine, methionine, and threonine

Amino acid	Organism	Concentration of standard solution (μg/ml)	Basal medium	
			Reference	% of recommended concentration
L-Lysine	*L. mesenteroides* P-60	1.00	8	33.3
L-Methionine	*L. mesenteroides* P-60	0.25	8	33.3
L-Threonine	*S. faecalis* 9790	0.50	14	33.3

The method was applied to plasma obtained from individual rats fed mixtures of foods containing bread and various supplementary proteins. The lysine content of the mixed diets was determined microbiologically (8). The protein-free basal diet of Chapman *et al.* (9) was supplemented with 10% protein supplied by whole egg or bread plus other proteins and given to 125-g male Wistar rats for 24 hours (eight animals per group). The animals were then killed and blood collected and assayed for lysine and methionine as outlined above. The relationship between plasma free lysine levels and lysine content of the food is shown in Fig. 3. A reasonably good relationship was found, despite considerable variability between animals given two of the diets. The average standard error of the mean for the seven groups was 4.0. The average plasma free methionine values (in μg/ml) for these groups were as follows:

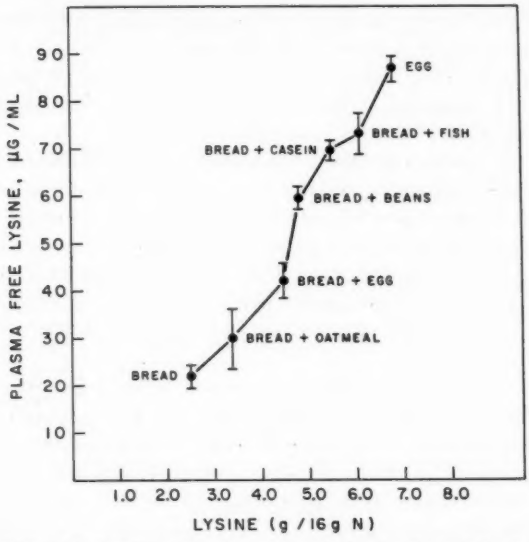

FIG. 3. Relationship between the plasma free lysine levels and the lysine content of the food.

bread, 5.5; bread plus oatmeal, 5.0; bread plus egg, 6.4; bread plus beans, 4.2; bread plus casein, 5.6; bread plus fish, 5.9; and egg, 8.6. There was a reasonably direct relationship also between the methionine content of the plasma and the methionine plus cystine scores for these mixtures of foods calculated from tables given previously (10).

Discussion

A study of the change in the absorbance of aqueous BTB solutions from the blue-green color (pH 6.8) to the yellow-green color (pH 6.4) showed that the most sensitive wavelength to measure the change in absorbance was at 610 mμ. The absorbance of a BTB solution decreased as the color changed from blue-green to yellow; in the assay the absorbance decreased as the lactic acid concentration increased. It was important therefore to keep growth to the lowest level practical although slight turbidity which occurred at the highest levels of the standard did not appear to interfere with the assay.

It was not surprising that the conditions for the lysine and methionine assays were similar since the same basal medium, with the exception of lysine and methionine content, and test organism were employed for both assays. The fact that the threonine assay, with a different basal medium and test organism, was readily modified using the same conditions as for the lysine and methionine assays suggests that the present method may be applicable to many common microbiological assays. The microassay for methionine should prove particularly useful because human serum frequently contains too little methionine for

an accurate estimation by conventional microbiological methods (11). On the basis of the present study it seems likely that microassays for other amino acids could be carried out, using dilute standard amino acid solutions containing 5% of the amount recommended in the original method and using the basal medium diluted to one third of the recommended amount.

To be useful in measuring blood free amino acid levels in human subjects, a method should be sensitive enough to measure several amino acids in the smallest volume of blood possible, preferably that obtainable by finger punch. Volumes of plasma as small as 0.04 ml have been deproteinized using very dilute solutions of sulphuric acid and sodium tungstate; a dilution of 1–30 occurred during preparation of the protein-free plasma. The lysine content of samples prepared in this way agreed very closely with samples prepared from 1.28 ml of plasma.

A preliminary study of the relation between plasma free lysine and lysine content of foodstuffs ingested confirms and extends the work of others (1, 2, 3, 4). The plasma lysine values found with egg are of particular interest. Since egg is generally considered to contain just sufficient lysine for optimal growth of rats (12) a plasma lysine level approximately the same as that produced by feeding egg at the 10% protein level (85 μg/ml) should contain sufficient lysine for optimal growth. Factors affecting the plasma free lysine concentration are considered in a subsequent paper (13).

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BLOOD AMINO ACID STUDIES

II. EFFECTS OF DIETARY LYSINE CONCENTRATION, SEX, AND GROWTH RATE ON PLASMA FREE LYSINE AND THREONINE LEVELS IN THE RAT[1]

A. B. MORRISON, E. J. MIDDLETON, AND J. M. MCLAUGHLAN

Abstract

Plasma free lysine levels of growing female rats given a lysine-deficient diet supplemented with graded amounts of lysine were higher than those of males. In both sexes, plasma free lysine values rose rapidly in response to added dietary lysine, and reached a maximum at a dietary lysine concentration of approximately 1.0%. This amount of dietary lysine was about 0.2% greater than that found necessary for maximum growth. Male rats given diets containing rapeseed oil and graded amounts of lysine showed reduced growth and lower plasma lysine and threonine levels as compared to animals given similar diets containing corn oil, but the amount of dietary lysine required for maximum plasma lysine levels was not influenced by the growth rate. Plasma free threonine levels showed a reciprocal relationship with those for lysine. It was concluded that measurement of the ratio between plasma free lysine and threonine levels may provide a sensitive indication of the adequacy of the dietary lysine content.

Introduction

Factors influencing plasma free amino acid levels in growing animals have not been studied extensively. Denton *et al.* (1, 2) observed that, in general, increases in plasma amino acid levels after a protein meal were proportional to the amounts of amino acid in the protein. Longenecker and Hause (3) reported more conclusive evidence that a direct relationship exists between changes in plasma amino acid levels after a meal and the amino acid composition of the ingested protein. They noted that plasma amino acid levels in adult dogs given meals containing 32% casein, wheat gluten, or gelatin were directly dependent upon the amino acid composition of the protein, if it was assumed that the amino acids were removed from the blood by the tissues at rates proportional to the amino acid requirements of the dog. Guggenheim *et al.* (4) gave test meals providing different proteins to rats and observed that the increases in the levels of lysine and methionine in the portal blood were correlated with the amounts, digestive release, and absorption of these amino acids. Recently, McLaughlan *et al.* (5) reported that blood free lysine levels of rats showed good correlation with the lysine content of the protein ingested during a 1-day test period. In studies with chicks, Gray *et al.* (6) found that animals given a lysine-deficient diet had lower free lysine and higher free threonine levels in the plasma than those given a diet containing adequate amounts of lysine.

The experiments reported herein were conducted as part of a project to study, systematically, factors influencing plasma free amino acid levels. A

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Contribution from the Food and Drugs Laboratories, Department of National Health and Welfare, Ottawa, Canada.

knowledge of these factors may be of great value in investigations on protein utilization in human subjects. The present studies were concerned with the effects of sex and growth rate on plasma free lysine levels in growing rats given various amounts of lysine in the diet. Interactions of plasma free lysine and threonine levels were also investigated briefly.

Experimental

Weanling Wistar rats of the Food and Drugs colony were housed individually in screened-bottom cages kept in an air-conditioned room maintained at 74° to 76° F. Food and water were supplied ad libitum, records kept of the amount of food consumed by each rat, and the animals were weighed individually at weekly intervals for 3 weeks. At the end of each experiment, the animals were anesthetized with ether, decapitated, and the blood was collected. Because of the reported effects of fasting on blood free amino acid levels (6, 7), the animals were not fasted. In experiment 1, individual samples were collected from each animal, but in experiment 2, the blood was pooled for each group in order to provide sufficient material on which to carry out threonine assays. Heparin was used as an anticoagulant. The samples were centrifuged, deproteinized by a slight modification of the procedure of Hier and Bergeim (8), and frozen until assayed. Lysine was determined in the protein-free filtrates by the microtechnique of McLaughlan *et al.* (5) and threonine was determined by microbiological procedures given by Block and Weiss (9).

The basal lysine-deficient diet used in the experiments was similar to that of Gupta *et al.* (10) and contained, in percentage, sucrose, 59.35; wheat gluten, 20; mineral mixture U.S.P. XIV, 4; corn oil, 10; vitamin mixture, 1; nonnutritive cellulose, 5; L-histidine HCl, 0.20; DL-methionine, 0.20; DL-threonine, 0.20; DL-tryptophan, 0.05. The diet contained 0.32% of lysine, by microbiological assay.

In experiment 1, groups of six male or six female rats received the basal diet alone or supplemented with various levels of lysine. Lysine was added as reagent grade L-lysine HCl, and replaced an equal amount of sucrose.

Experiment 2 was conducted to determine the effect of growth rate on plasma free lysine levels and to investigate briefly the interrelationship between plasma free lysine and threonine levels. In this experiment, the basal diet was modified to contain 20% corn oil, by removal of 10% sucrose. Groups of six male rats received this diet alone or supplemented with graded amounts of lysine. Other groups received similar diets containing 20% Golden rapeseed oil, instead of corn oil. Beare *et al.* (11) showed previously that at this level in the diet, rapeseed oil significantly inhibits growth, because of its content of erucic acid.

The results of the experiments were analyzed statistically by methods outlined by Snedecor (12).

Results and Discussion

The values for plasma lysine found in experiment 1 are summarized in Fig. 1A and growth curves are given in Fig. 1B. Weight gains adjusted by

covariance analysis for differences in food consumption were plotted against the log of the dose, as suggested by Almquist (13). Lines of best fit were drawn after visual inspection of the data. The results of the lysine determinations showed that after an initial lag, plasma free lysine levels rose rapidly in response to added dietary lysine. In both sexes, the level reached a maximum when the diet contained approximately 1.0% of lysine. Considerable individual variation in plasma free lysine levels was observed. For the various groups, coefficients of variation ranged from 11% to 38% in the males, and from 11% to 49% in the females. The sex of the test animal significantly influenced the plasma levels found. With the exception of the animals given the basal diet, females showed higher values than males. Maximum plasma free lysine values were found at 130 μg/ml in the males and at 171 μg/ml in the females. These results are in agreement with those of Wheeler and Morgan (14), who found higher levels of free lysine in the portal blood of female rats than in that of males given raw or autoclaved fresh pork, and are probably related to the effects of growth hormone on amino acid transport into tissues (15).

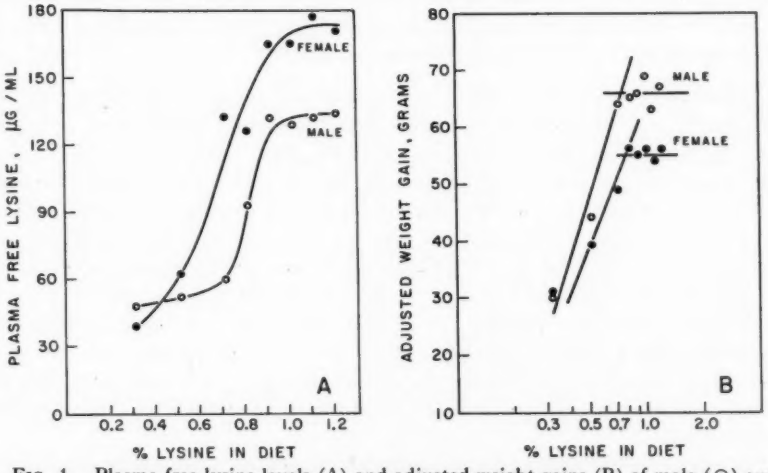

FIG. 1. Plasma free lysine levels (A) and adjusted weight gains (B) of male (○) and female (●) rats given diets containing different amounts of lysine.

The results of the growth studies conducted in experiment 1 (Fig. 1B) indicated that both sexes showed maximum growth at a dietary lysine concentration of approximately 0.80% of the diet. This value was approximately 0.20% lower than that required for maximum plasma lysine levels. In contrast to results reported by Rosenberg and Culik (16), no difference was noted in the lysine requirement of the two sexes, whether measured by growth rate or plasma free lysine levels.

The results of experiment 2, conducted to study lysine and threonine levels in the plasma of animals receiving graded amounts of lysine in the presence

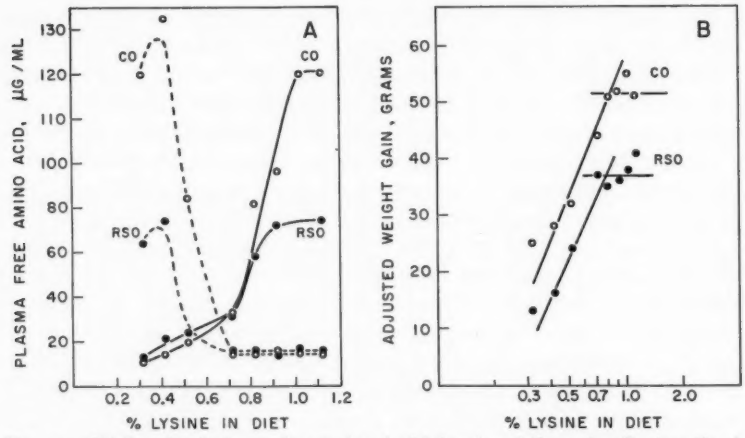

FIG. 2. (A) Levels of plasma free lysine (solid lines) and threonine (broken lines) in male rats given diets containing corn oil (CO) or rapeseed oil (RSO) and varying amounts of lysine.

(B) Adjusted weight gains of male rats given diets containing corn oil (CO) or rapeseed oil (RSO) and varying amounts of lysine.

and absence of a growth inhibitor, are summarized in Fig. 2. In this experiment, the growth rate was altered by inclusion in the diet of rapeseed oil, which has been shown to depress growth by reducing food consumption (11). As in experiment 1, plasma free lysine values showed an initial slow response to added dietary lysine, and then increased very rapidly as the dietary lysine level was raised further (Fig. 2A). Although the maximum growth rate obtained with rapeseed oil was approximately 35% less than that found with corn oil (Fig. 2B), the growth inhibition had no effect on the percentage of dietary lysine required to produce maximum plasma free lysine values or maximum growth. The maximum plasma lysine value obtained with rapeseed oil, however, was much lower than that found with corn oil. The reason for this is unknown, but may be related, in part, to the reduced food consumption of the animals given rapeseed oil.

Plasma free threonine values (Fig. 2A) showed a reciprocal relationship with those for free lysine. When dietary lysine was low, and plasma lysine values were depressed, plasma threonine values were high. As plasma lysine levels increased in response to added dietary lysine, plasma threonine levels showed an initial slight rise and then fell rapidly, until they levelled out and stayed constant at dietary lysine concentrations of 0.72% or more. At low levels of dietary lysine, plasma threonine values were much lower in animals given rapeseed oil than in those given corn oil. However, the minimum threonine levels found with animals given 0.72% or more of dietary lysine were not significantly influenced by the dietary fat.

The present findings on the reciprocal relationship between free lysine and

threonine levels in the plasma confirm and extend those of Gray *et al.* (6), who found high free threonine levels in the plasma of lysine-deficient chicks. As suggested by these workers, the elevated threonine level in the plasma of animals given lysine-deficient diets may be related, in part, to reduced tissue demands for protein synthesis. If threonine has a relatively slow rate of turnover in vivo, it might then accumulate in the blood. This suggestion is supported by the observation that plasma threonine levels reached a minimum when the dietary lysine concentration was high enough to permit maximal growth, i.e. when demands for tissue synthesis were highest.

Until lysine ceased to be the factor limiting growth, plasma free lysine levels were much less influenced by dietary lysine concentration than were weight gains. Because of this fact, measurement of plasma free lysine levels alone may provide a misleading estimate of the adequacy of the dietary lysine content. Measurement of the ratio between the levels of free plasma lysine and threonine, however, may provide a very sensitive indicator of the status of lysine nutrition. Lysine/threonine ratios of much less than 1 were found when the dietary lysine concentration was low. When the lysine content of the diet was approximately adequate for growth, the lysine/threonine ratio approached unity, whereas when lysine was present in the diet in amounts greater than that required for adequate growth, the lysine/threonine ratio was much greater than 1.

The plasma free lysine level obtained when growth was highest was found in these studies to be approximately 90 μg/ml for male rats. It is of interest to note that this value is very similar to that found by McLaughlan *et al.* (5) with 10% egg protein, which is almost perfectly balanced in terms of the lysine requirement of the growing rat.

Although the present studies were concerned primarily with plasma amino acid levels, the results of both experiments indicated that the rate of growth did not significantly influence the lysine requirement, as measured by plasma free lysine levels or weight gain. This finding confirms that of Griminger and Scott (17), but is in disagreement with the contention of Edwards *et al.* (18). As has been pointed out previously (17) the basal diet used by the latter workers may have failed to provide ample amounts of balanced protein.

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BLOOD AMINO ACID STUDIES

III. EFFECTS OF AMOUNT AND QUALITY OF DIETARY PROTEIN AND LENGTH OF TEST PERIOD ON PLASMA FREE LYSINE LEVELS IN THE RAT[1]

A. B. MORRISON, J. M. MCLAUGHLAN, F. J. NOEL, AND J. A. CAMPBELL

Abstract

Male rats were given diets containing 10 or 20% protein from fish flour or wheat gluten plus histidine, methionine, threonine, and tryptophan, and plasma free lysine levels were determined after 3 and 7 days. The levels dropped by approximately 50% from initial values when the diet contained gluten and rose by approximately 100% when the diet contained fish flour. The protein level of the diet had no significant effect on plasma free lysine levels. Values found after 3 days were similar to those found after 7 days.

In further studies, male rats were fasted for 19 hours and given diets containing 10% protein from bread or fish flour for 2 hours, 1 day, or 3 days. No marked effects of diet on plasma free lysine values were found after 2 hours, but after 1 or 3 days values found with bread were much lower than those with fish flour. Plasma free lysine levels of animals given heat-damaged casein or soy flour were lower than those of animals given unheated proteins. It was concluded that plamsa free lysine levels reflect the amount and availability of lysine in the food.

Introduction

Although it has been established that, in general, the levels of free amino acids in the blood reflect those in the ingested protein, few attempts have been made to study, systematically, factors influencing blood amino acid levels. In previous reports from this laboratory (1, 2) it was shown that plasma free lysine levels in growing rats were significantly influenced by sex, growth rate, and dietary lysine concentration. The present report deals with studies on the effects of amount and quality of dietary protein and length of the test period on plasma free lysine levels in the rat.

Experimental and Results

Male Wistar rats of the Food and Drugs colony, averaging 125 g in weight, were used in the experiments. The animals received a standard laboratory diet (Master Fox Cubes*) from weaning until they were selected for the studies. The rats were housed individually in screened-bottom cages kept in an air-conditioned room maintained at 74° to 76° F. The basal diet used in the studies was similar to that of Chapman *et al.* (3), and contained, in percentage, sucrose, 80; corn oil, 10; U.S.P. XIV mineral mixture, 4; non-nutritive cellulose, 5; and vitamin mixture, 1. The various proteins tested were added to the basal diet at the expense of carbohydrate. Sucrose was used as the carbohydrate, instead of cornstarch, because of reports that the dietary carbohydrate may influence amino acid utilization (4, 5).

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Contribution from the Food and Drugs Laboratories, Department of National Health and Welfare, Ottawa, Canada.

*Toronto Elevators, Toronto, Ontario.

Experiment 1

This experiment was conducted to study the effects of level of protein in the diet, and length of the test period, on plasma free lysine levels. Nine animals were killed initially to provide baseline values. Comparable groups of five rats then received the basal diet supplemented with 10 or 20% protein (N×6.25), supplied by wheat gluten or fish flour. The fish flour was a sample shown previously (6) to be of high nutritional value. Because of the multiple amino acid deficiencies of wheat gluten, the diet containing 10% gluten protein was supplemented with the following amino acids, in percentage: L-histidine.HCl, 0.10; DL-methionine, 0.10; DL-threonine, 0.10; DL-tryptophan, 0.025. Twice these amounts of supplementary amino acids were added to the diet containing 20% gluten protein. The diets were given to the animals for 3 or 7 days on a pair-fed basis, so that within each protein level and time interval, the food consumption of the animals given fish flour was limited to that of pair-mates given wheat gluten. After the time intervals specified, the animals were anesthetized with ether, decapitated, and the blood of each rat was collected separately in heparinized tubes. Plasma free lysine levels were determined by procedures described previously (1).

The results are summarized in Table I. Missing values indicate samples lost for technical reasons. The mean plasma free lysine value for the control animals was 47 μg/ml. Within 3 days after the gluten diet was given, the value dropped

TABLE I

Plasma free lysine levels (μg/ml) of male rats given diets containing 10 or 20% protein from fish flour or wheat gluten plus amino acids for 3 or 7 days

	3 days				7 days			
	10% protein from:		20% protein from:		10% protein from:		20% protein from:	
Control animals	Wheat gluten	Fish flour	Wheat gluten	Fish flour	Wheat gluten	Fish flour	Wheat gluten	Fish flour
42	23	76	30	94	24	103	32	96
44	35	78	15	73	20	—	17	96
45	19	—	18	109	18	84	22	—
56	17	60	16	103	20	84	21	92
47	19	80	32	108	12	104	20	79
44								
48								
49								
51								
Mean 47	23	74	22	97	19	93	22	91

by approximately 50%, and showed an approximate 100% increase when the dietary protein was supplied by fish flour. At both time intervals and protein levels tested, the values found for wheat gluten were much lower than those for fish flour. The dietary protein level had no significant effect on plasma free lysine values found with either gluten or fish flour. Values found after 3 days were similar to those found after 7 days.

Experiment 2

The effects of length of the test period were investigated further in experiment 2. Five rats were killed initially to provide baseline values for blood lysine levels. The remaining animals were fasted for 19 hours, and five rats were sacrificed to provide fasting control values. Fifteen comparable pairs of animals were then selected. Five pairs were given the basal diet supplemented with 10% protein (N×6.25) supplied by white bread or fish flour, for 2 hours, 1 day, or 3 days. The animals were fed ad libitum and records were kept of food consumption. At the end of the test periods, the animals were killed, blood was collected and analyzed for plasma free lysine as in experiment 1.

The results (Table II) showed that plasma lysine levels were somewhat higher in fasted animals than in those which received food ad libitum until they were killed. Plasma free lysine levels of the animals given the diet containing

TABLE II

Effect of length of test period on plasma free lysine levels (μg/ml) in rats given diets containing white bread or fish flour

	Control		Diets given for:					
			2 hours		1 day		3 days	
	Fed	Fasted	Bread	Fish flour	Bread	Fish flour	Bread	Fish flour
	57	67	44	53	22	130	30	103
	51	62	41	56	11	86	38	107
	46	60	35	58	12	110	25	144
	50	60	35	64	16	117	24	138
	45	56	41	60	30	105	24	122
Mean	50	61	39	58	18	110	28	123

bread began to decrease within 2 hours, and those of the animals given the diet containing fish flour began to increase. The difference between the two diets was not marked after 2 hours, but was very large after 1 or 3 days. The values found after 1 day were not markedly different from those found after 3 days. Considerable within-diet variation was observed in this study, as in the others.

Experiment 3

This experiment was conducted to determine whether plasma free lysine levels reflect the biological availability of lysine in the ingested protein. Comparable groups of eight rats were fasted for 19 hours and then received the basal diet supplemented with 10% protein (N×6.25) from casein or soy flour. Other groups received similar diets containing casein or soy flour autoclaved in 1-in. layers in metal pans for 3 hours at 15 p.s.i. This amount of heat treatment is known to reduce markedly the biological availability of amino acids in milk or soy proteins (7, 8). The animals were given the diets ad libitum for 22 hours and killed 2 hours later. Plasma free lysine levels were determined as in the first two experiments. The biological value of the heated and unheated proteins

was determined by the protein efficiency ratio (P.E.R.) procedure of Chapman *et al.* (3), using a 2-week assay period.

The results (Table III) showed that heat treatment of casein and soy flour markedly reduced their nutritional value, as measured by P.E.R. assay. The effects of autoclaving on soy flour were particularly severe, reducing P.E.R.

TABLE III

Plasma free lysine and protein efficiency ratio (P.E.R.) values found with heated and unheated proteins

Protein	Plasma free lysine (μg/ml)	P.E.R. (2 weeks) (g gain/g protein)
Casein	115 ± 8.8*	3.39 ± 0.09
Heated casein	97.5 ± 3.8	2.62 ± 0.10
Soy flour	82 ± 2.8	2.66 ± 0.17
Heated soy flour	72 ± 3.6	0.86 ± 0.09

*Standard error.

from 2.66 to 0.86. The heated casein was less severely damaged. Plasma free lysine levels were somewhat lower in the animals given heat-damaged proteins than in those which received unheated casein or soy flour. The differences, however, were not marked, although statistically significant. The 2-hour fast before the animals were killed did not appear to reduce variability between rats.

Discussion

The results of these studies show that by use of the microtechnique of McLaughlan *et al.* (1), it is possible to determine the effects of different proteins on plasma free lysine levels in individual rats fed relatively low protein diets for short lengths of time. Marked differences were found in the plasma free lysine levels of animals fed diets containing adequate or deficient amounts of lysine. The extent of the differences was related to the amounts of lysine in the ingested protein. Values found after 1 day were comparable to those obtained when the animals had received the diets for 1 week. Increasing the protein level of the diet from 10 to 20% had no significant effect on plasma free lysine levels. This finding may be explained, in part, by greater protein synthesis at the higher dietary protein level, resulting in an increased rate of removal of lysine from the blood by the tissues, with the net result that plasma free lysine levels remained unchanged.

It is of interest to note that fasting for 19 hours appeared somewhat to increase plasma free lysine values. This finding is in agreement with the results of Henderson *et al.* (9), who also observed increases in the plasma levels of other free amino acids after rats were subjected to short fasts. In the chick, plasma free lysine levels are also elevated by fasting (10, 11) but, in the human, fasting has been reported to decrease plasma free lysine levels (12).

No marked effects of dietary protein on plasma free lysine levels were observed after a single meal. These results do not agree with those of Longenecker

and Hause (13), or Guggenheim *et al.* (5), who noted significant changes in plasma free amino acid levels within a short period after giving single meals of test protein. The experimental procedures used by these two groups of investigators, however, were not the same as those used in the present studies. Longenecker and Hause (13) gave meals containing 32% protein, which were spoon-fed if the dog did not eat all of the meal within a few minutes. Changes in plasma amino acid levels after ingestion of single meals containing 16% protein were found by these workers to be irregular. Guggenheim *et al.* (5) force-fed rats with an aqueous suspension of 5% protein and 5% carbohydrate. Although the present studies did not show marked changes in plasma free lysine levels after a single meal, they demonstrated that it is not necessary to use force-feeding or high-protein diets in order to show the effects of different proteins on plasma free lysine levels. It is possible therefore to evaluate cereals and mixed diets of relatively low protein content by the procedures outlined herein.

The results of the present studies and of those reported by Longenecker and Hause (13) showed a drop in plasma free lysine levels after ingestion of proteins deficient in lysine. This phenomenon was not observed by Guggenheim *et al.* (5), who sampled portal blood, the amino acid levels of which had not reached equilibrium with the tissues.

It is probable that the differences in plasma free lysine levels found with heated and unheated proteins in experiment 3 were partially obscured by the 2-hour fast before the animals were killed. Guggenheim *et al.* (5) found that plasma levels of lysine and methionine approached the control level 180 minutes after ingestion of a test meal.

In view of the correlation observed between plasma free amino acid levels and the amino acid composition of the ingested protein, it is pertinent to ask whether measurement of blood amino acid levels provides a basis for evaluating the nutritional adequacy of proteins. Preliminary evidence suggests that the method is of value, but may not possess marked advantages over conventional rat bio-assay procedures. Perhaps the greatest value of these studies may be in providing background information for the application of this technique to studies on protein utilization in man. This problem is now being investigated.

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ESTIMATION OF THE BIOLOGICAL POTENCY OF VARIOUS FORMS OF VITAMIN A[1]

T. K. MURRAY AND J. A. CAMPBELL

Abstract

The low biological potency of 9-*cis* and 9,13-di-*cis* vitamin A, heretofore measured by growth and liver storage assays, has been confirmed by vaginal smear assays. Appreciable quantities of these isomers in pharmaceuticals would lead to overestimation of biological potency by the U.S.P. XV or the antimony trichloride methods. In an extensive series of assays the U.S.P. method accurately estimated the biological potency of most oil-soluble, water-dispersed, and dry, stabilized samples of vitamin A. The antimony trichloride method did not accurately estimate biological potency but, for many samples, correction of these values in accordance with their maleic values was not justified. Water-dispersed and dry, stabilized samples of vitamin A were not better utilized than oil solutions of the vitamin.

Lehman *et al.* (1) recently reported that both the U.S.P. XV spectrophotometric and the antimony trichloride colorimetric procedures overestimated the biological potency of vitamin A in stored aqueous multivitamin preparations. This situation was attributed to the presence of low potency *cis* isomers of vitamin A. As these authors indicated, the U.S.P. XV method accurately measures the biological potency of all-*trans* and 13-mono-*cis* (neo) vitamin A but overevaluates 9-mono-*cis* and 9,13-di-*cis* vitamin A. The antimony trichloride method overestimates the biological potency of all of the *cis* isomers. Ames, Swanson, and Lehman (2) proposed that a measure of the biological potency of aqueous, multivitamin preparations and of chemically isomerized vitamin A palmitate could best be obtained by correction of the antimony trichloride values with a cubic regression equation based on the maleic value of the sample. The maleic value gives a measure of the combined 13-mono-*cis* and 9,13-di-*cis* content and, by difference, the combined all-*trans* and 9-mono-*cis* fraction. These authors suggested that this method might have general application to mixtures of vitamin A isomers from both synthetic and natural sources.

The procedure of Ames *et al.* (2) was studied by De Ritter (3) and found to apply to some but not all water-dispersed samples. He proposed instead an empirical correction of the U.S.P. XV method based on the maleic value of the sample.

The validity of these corrections rests on acceptance of published biological potencies of the *cis* isomers of vitamin A and on the theory that the 9-*cis* isomers occur, or are formed, in amounts proportional to the maleic value. The fact that two different correction procedures have been proposed indicates that these factors are in doubt. Murray and Campbell (4) published evidence that the spectrophotometric assay described in U.S.P. XIV (5) overestimated biological

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potency by 8% but modifications made in the U.S.P. XV method (6) result in somewhat lower values which would more closely approximate biological potency.

If a method is to apply to a variety of samples, factors other than isomer content must be considered. Water-dispersed vitamin A was reported (7, 8) to be better utilized than vitamin A in oil but De Ritter (3), in his experiments, assayed water-dispersed samples against the standard in oil. Ames *et al.* (2) administered both sample and standard in oil after saponification.

Dry, stabilized forms of vitamin A were also reported (9, 10) to be better utilized than oil solutions of the vitamins. Bauernfeind and De Ritter (11) studied one dry preparation and found it superior to an oil dose only in the few hours immediately following administration.

It was of interest to determine if the proposed corrections of Ames and De Ritter could be applied to all pharmaceutical products containing vitamin A. Consequently the biological potency of various samples was compared with the values obtained spectrophotometrically and colorimetrically. The following is an account of these studies.

Methods

Vitamin A was estimated spectrophotometrically by the U.S.P. XV (6)* method and colorimetrically by the antimony trichloride method most recently described by Ames and Lehman (12). Maleic values were obtained by the method of Robeson and Baxter (13). Biological potency was determined by the vaginal smear assay of Pugsley, Wills, and Crandall (14) and the liver storage method of Ames and Harris (15). Doses were dissolved in corn oil or dispersed in water with the aid of 20% Tween 80. Dry vitamin A preparations were mixed with a few grams of feed and offered to rats which had been fasted overnight. Spillage was returned to the feed dish until the whole dose was consumed, usually in a few hours. In these assays the standard was mixed with the food just before dosing and offered to the rat in the same way as were the dry samples. Utilization of the standard given in this way was the same as with the usual oral dose.

Results and Discussion

Biological Potency of Cis Isomers

The biological potencies of various *cis* isomers of vitamin A acetate and aldehyde were determined by the vaginal smear assay. These results, compared with those obtained by Ames *et al.* (16, 17) by growth and liver storage methods, are shown in Table I. Vitamin A aldehydes are not encountered in commercial samples but their assay provided a valid comparison of the results obtained by different methods in different laboratories. Clearly there was good agreement between the two laboratories and between methods. The potency of the 9,13-di-*cis* isomer measured in this laboratory seemed somewhat low but the antimony trichloride value was also low, and the relative biopotency (biopotency ×

*The same procedure appears in U.S.P. XVI.

TABLE I

Biological potency of isomers of vitamin A

Isomer	Form		Biological assay: Vaginal smear (I.U./g)	Biological assay: Fiducial limits (%)	Biological assay: Growth and liver storage* (I.U./g)
All-*trans*	Acetate		2,907,000		2,907,000
All-*trans*	Aldehyde		3,000,000	±5	3,050,000
2-Mono-*cis*	Acetate		1,960,000		
			2,040,000		
			2,000,000		
		Mean	2,000,000	±9	2,190,000
2-Mono-*cis*	Aldehyde		3,230,000	±12	3,120,000
6-Mono-*cis*	Acetate		614,000		634,000
6-Mono-*cis*	Aldehyde		699,000		
			692,000		
		Mean	695,000	±8	637,000
2,6-Di-*cis*	Acetate		509,000	±13	688,000

*Ames *et al.* (16, 17)

100/$SBCl_3$) of 24.6% agreed well with the results reported by Ames *et al.* (16). The physical and chemical characteristics of the other samples, as well as the biological potencies, corresponded with those published by others (16, 17). It follows that the presence of appreciable amounts of 9-*cis* and 9,13-di-*cis* vitamin A in commercial samples would result in an overestimation of biological potency by both the U.S.P. XV and antimony trichloride methods.

Estimation of Biopotency

Figures 1 and 2 contain the relative biopotencies (biopotency×100/U.S.P. XV or $SBCl_3$) of oil and water-dispersed samples. The U.S.P. XV method overestimated biological potency of both types of samples by 2% while the antimony trichloride values exceeded the biological potency of oil samples by 17%, water-dispersed samples by 12%. These results, which agreed with earlier work from this laboratory (4), gave no indication that U.S.P. XV values should be corrected as suggested by De Ritter (3).

The relationship of relative biopotencies, maleic values, and the cubic regression equation of Ames *et al.* (2) are shown in Fig. 3. The relative biopotencies of certain samples, including a pre-isomerized concentrate and a sample of rat-liver oil known to contain (18) low-potency *cis* isomers, fell close to the regression line but other samples did not. Thus, while Ames *et al.* (2) have demonstrated the usefulness of the cubic regression equation in estimating the biological potency of certain preparations this equation cannot be applied indiscriminately to all vitamin A products.

Water-dispersed Vitamin A

Table II contains the results of two liver storage and two vaginal smear assays in which oil and water-dispersed samples of vitamin A were compared. Over

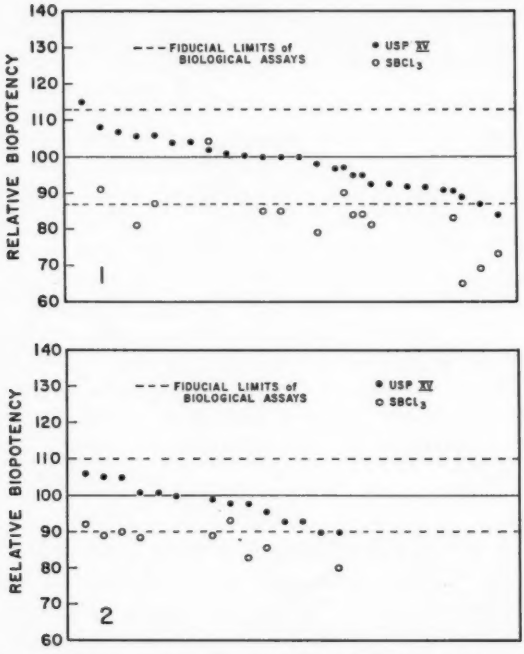

FIG. 1. Relative biopotency (bio-assay×100/U.S.P. XV and bio-assay×100/$SbCl_3$) of oil solutions of vitamin A.

FIG. 2. Relative biopotency (bio-assay×100/U.S.P. XV and bio-assay×100/$SbCl_3$) of water-dispersed samples of vitamin A.

the wide dosing range covered (25–2000 I.U.) there was little difference in utilization. This is in line with De Ritter's experience (3) and justifies the comparison of water-dispersed doses with the standard in oil.

TABLE II

Utilization of oil and water doses of vitamin A

		Biological potency			
Sample	Assay	Oil dose (I.U./g)	Water dose (I.U./g)	Fiducial limits (%)	Water dose × 100 / Oil dose (%)
1	Vaginal smear	1,130,000	1,150,000	±6	102
2	Vaginal smear	100,000	94,200	±19	94
3	Liver storage	100,000	105,000	±7	105
4	Liver storage	100,000	110,000	±5	110
				Mean	103

Dry Vitamin A

The results of physical, chemical, and biological assays of various dry vitamin A products are shown in Table III and Fig. 4. The U.S.P. XV method

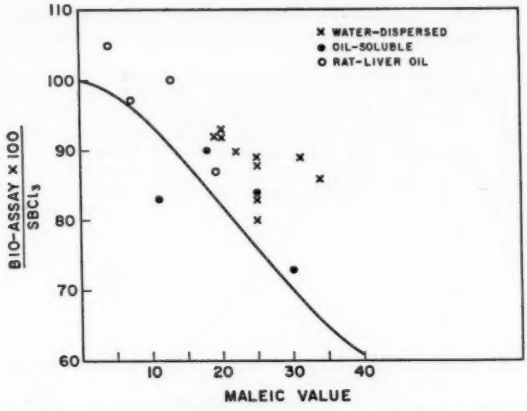

FIG. 3. Relative biopotency of various samples of vitamin A in relation to their maleic value and the cubic regression line of Ames *et al.* (2).

TABLE III

Biological potency of dry vitamin A estimated by various methods

Sample	Maleic value (%)	Potency estimated by: U.S.P. XV (I.U./g)	Potency estimated by: $SbCl_3$ (I.U./g)	Potency estimated by: Bio-assay (I.U./g)	Relative biopotency: Bio-assay × 100 / U.S.P. XV	Relative biopotency: Bio-assay × 100 / $SbCl_3$
1	0	279,000	303,000	273,000	98	90
2	3	239,000	245,000	249,000	104	102
3	4	355,000	366,000	364,000	103	99
4	19	212,000	237,000	219,000 213,000		
				Mean 216,000	102	91
5	27	207,000	249,000	196,000 193,000		
				Mean 195,000	94	78
6	28	171,000	214,000	156,000 149,000		
				Mean 152,000	89	71
					Mean 98	88

estimated biological potency to within 2%, an observation which indicated that, when stability was not a factor, vitamin A was not better utilized from these products than from oil. This was similar to the experience of Bauernfeind and De Ritter (11), who studied a single dry product. The antimony trichloride method overestimated biological potency by 12% as found with oil and water-dispersed samples but in the dry product there was evidence (Fig. 4) that the relative biopotencies followed the cubic regression line and it seems likely that 9-*cis* isomers of vitamin A were present.

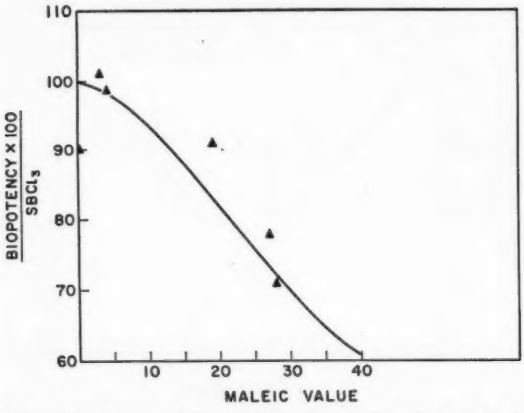

FIG. 4. Relative biopotency of dry, stabilized samples of vitamin A in relation to their maleic value and the cubic regression line of Ames *et al.* (2).

It was concluded that for the assay of a variety of vitamin A samples the U.S.P. XV method gave, on the average, a good estimate of biological potency. Low-potency *cis* isomers undoubtedly occurred in some samples and were a potential source of error but their presence was not always accurately reflected by the maleic values and corrections based on these values should not be applied to samples of unknown composition. A simple and accurate method for the direct determination of 9-*cis* isomers is required. The utilization of water-dispersed and dry, stabilized vitamin A was not superior to that of oil solutions.

Acknowledgments

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ON THE NUCLEOTIDE SPECIFICITY OF THE POLYNUCLEOTIDE PHOSPHORYLASE OF THE CROWN-GALL TUMOR-INDUCING ORGANISM AGROBACTERIUM TUMEFACIENS[1]

A. VARDANIS[2] AND R. M. HOCHSTER

Abstract

An investigation of the nucleotide specificity of the polynucleotide phosphorylase of *Agrobacterium tumefaciens* was undertaken, using the measurable increase in viscosity as an index of activity. It was found that ADP and CDP were polymerized readily and at approximately equal rates. The enzyme exhibited more moderate activity with UDP and was completely inactive with GDP. The ineffectiveness of the enzyme with mixtures of all four ribonucleoside diphosphates was traced to the ability of GDP to act as an inhibitor in the polymerization of the other diphosphates. Evidence is presented to show that the inhibition of poly A synthesis effected by GDP is competitive. On the basis of the results obtained it is concluded that the polynucleotide phosphorylase is not likely to be responsible for RNA synthesis in *A. tumefaciens*.

Introduction

The study of the metabolic characteristics of *Agrobacterium tumefaciens* in this laboratory is aimed ultimately at the elucidation of the process of tumorogenesis in plants, as initiated by this organism. Even though much work has been reported in the past comparing normal and crown-gall tissue, little is known regarding the fundamental biochemistry of the inciting organism itself. A systematic investigation of key metabolic systems is expected to provide background information, with the aid of which a theory of the genesis of crown-gall tumor disease may be formulated. Moreover, it is hoped that such a study may reveal valuable clues regarding the nature of the tumor-inducing principle, the as yet unknown bacterial product believed to be responsible for the transformation of normal plant cells into crown-gall tumor cells (1, 2).

The presence and unusually high activity of polynucleotide phosphorylase in crude extracts of *A. tumefaciens* have been described previously (3). Since the initial discovery of this enzyme in *Azotobacter vinelandii* (4) a number of investigators have studied its nucleotide specificity. Thus, Grunberg-Manago *et al.* (5) found that when the enzyme was incubated with individual nucleoside diphosphates, and the reaction measured by means of P^{32} exchange and P_i release, all except GDP* showed ready conversion to the corresponding poly-

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[2]National Research Council of Canada Fellow in collaboration with the Canada Department of Agriculture, 1959–61.

*The following abbreviations are used: ADP, adenosine diphosphate; CDP, cytidine diphosphate; UDP, uridine diphosphate; GTP, guanosine triphosphate; GDP, guanosine diphosphate; GMP, guanosine monophosphate (guanylic acid); poly A, polyadenylic acid; poly C, polycytidylic acid; poly G, polyguanylic acid; poly AGUC, copolymer of adenylic, guanylic, uridylic, and cytidylic acids; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; P_i, inorganic orthophosphate; EDTA, ethylenediamine tetraacetate; Tris, tris(hydroxymethyl)aminomethane; and DEAE cellulose, diethylaminoethyl cellulose.

nucleotide. When mixed with other nucleoside diphosphates, however, GDP was utilized and polymers such as poly AGUC were formed (5). The synthesis of poly G was studied further by Singer *et al.* (6), who reported that the inclusion in the incubation medium of an oligoribonucleotide, containing an unsubstituted hydroxyl group at carbon 3′ of the terminal nucleoside residue, effected polymerization of GDP by serving as a primer on to which GMP units were added. On the other hand, they were able (7) to demonstrate an exchange between GDP and P_i^{32} analogous to the one cited with the other nucleoside diphosphates by using more favorable Mg^{++} and P_i concentrations in the absence of a primer. As a result, they suggested that the exchange reaction is independent of the polymerization process.

A previous speculation on the possible importance of polynucleotide phosphorylase in intracellular biosynthesis (3) has recently been underscored by the publication of evidence that polynucleotide phosphorylase may be intimately associated with the biosynthesis of β-galactosidase in *Escherichia coli* (8, 9).

It is the purpose of this paper to present the results of recent experiments on the nucleotide specificity of the polynucleotide phosphorylase in *A. tumefaciens* which form part of a study on RNA biosynthesis by this tumor-inducing organism.

Materials and Methods

Materials

The following substances used in this investigation were commercial preparations: ADP (Sigma Chemical Co. and Pabst Brewing Co.); CDP, UDP (Sigma Chemical Co.); GDP (Sigma Chemical Co. and California Corporation for Biochemical Research); and Tris (Sigma Chemical Co.).

Strains A_6 and B_6 of *A. tumefaciens* were originally obtained from Dr. A. C. Braun, Rockefeller Institute for Medical Research, New York. They were maintained on yeast extract – glucose–agar–$CaCO_3$ slants.

Enzyme Preparations

Bacteria were grown in a medium consisting of 1% yeast extract and 1% glucose in Fernbach flasks on a shaker for 18 hours. The cells were harvested, washed, and treated in the 10-kc Raytheon oscillator as previously described (10). The "whole extract" thus obtained was used as such without dialysis. Results given in this paper were obtained with strain B_6 of *A. tumefaciens* unless indicated otherwise. For the experiments reported in Fig. 2, a partially purified preparation of polynucleotide phosphorylase of strain A_6 was used. The latter preparation was kindly given to us by Dr. N. B. Madsen of this Institute and was obtained in the course of the purification of another enzyme. The experimental details of the purification procedure were as follows: The extract was prepared as indicated above with the exception of the buffer which was 0.01 *M* Tris – 0.005 *M* mercaptoethanol – 0.001 *M* EDTA (pH 7.5). A 0.35–0.60 saturated $(NH_4)_2SO_4$ fraction was obtained, dissolved in a small

volume of the above buffer, and dialyzed against more of the same buffer. This fraction (containing 1400 mg protein) was then transferred to a DEAE cellulose column (2×20 cm), containing 10 g DEAE cellulose previously treated with NaOH and washed successively with water and buffer. After flushing the column again with buffer, a salt gradient was established by using a reservoir of the buffer containing 0.5 *M* NaCl and a mixing chamber containing 300 ml of buffer alone. Fractions were collected automatically at 20-minute intervals and at a flow rate of 36 ml per hour. Polynucleotide phosphorylase activity was found in eight fractions collected between a calculated concentration of 0.27–0.34 *M* NaCl. The fraction showing the highest activity was dialyzed against buffer and used for the experiments reported in Fig. 2. All manipulations were carried out at 2°–4° C. A 19-fold purification of the enzyme was thus obtained as assayed by the system described below (see Viscometry).

Viscometry

The assay methods used most commonly for the determination of polynucleotide phosphorylase activity are: (*a*) P_i release, (*b*) P_i^{32} exchange, and (*c*) measurement of increase in viscosity. Since phosphatase activities could easily result in erroneously high P_i values this method was not adopted. The existing evidence in the literature, showing that P_i^{32} exchange appears to be independent of polymerization (7), suggested that a more direct method should be used. Inasmuch as measurement of increase in specific viscosity is related to true polymerization (i.e. to the long-chain molecules of the type sought in this investigation), the latter method was chosen. Specific viscosity was calculated in the manner described (3). For the experiments with the whole extract (crude enzyme preparation), miniature Ostwald–Fenske viscometers especially constructed for us by the Wakefield Company, Montreal, were used. They had an operating volume of 2 ml and an outflow time for water of 6 to 8 seconds. Specific components of the incubation mixtures used are described in the text.

In the assay system used for the determination of enzyme activity during the purification procedure, commercially available Ostwald–Fenske viscometers (Fisher No. 200) were employed. The reaction mixture in this test system consisted of: glycine–NaOH buffer (pH 9.5), 400 μmoles; $MgCl_2$, 30 μmoles; ADP, 100 μmoles; and enzyme in a total volume of 7 ml. A unit of enzyme was defined as the amount necessary to bring about an increase in specific viscosity of 0.1 during the first 10 minutes of reaction. The observed specific viscosity, under the conditions of the assay system, was directly proportional to the amount of enzyme added when $(\eta/\eta_0)-1 = 0.1$ to 0.7. The specific activity of the crude extract used for purification was 0.78 and that of the fraction used for the experiments reported in Fig. 2 was 14.6.

All experiments were carried out at 30° C. Reaction mixtures were preincubated for 5 minutes prior to the addition of the substrate(s) and a zero-time reading was taken within 20 seconds after this addition.

Analytical Methods

Protein concentration was routinely estimated by the spectrophotometric method of Warburg and Christian (11).

Experimental and Results

Polynucleotide phosphorylase activity of the crude extracts of *A. tumefaciens* was found to be quite stable. There was no detectable loss of activity for at least 5 days after the preparation of the extract providing it was stored at a temperature of 0–4° C. Considerable variation was observed, however, between different preparations. For the latter reason the series of experiments reported in each individual table or figure were carried out with the same extract.

The optimum conditions of incubation with the crude extract were established by two series of experiments at different pH's and with different Mg^{++} concentrations, utilizing each of the four nucleoside diphosphates (ADP, CDP, UDP, GDP) as substrate. The resultant pH activity curve is given in Fig. 1.

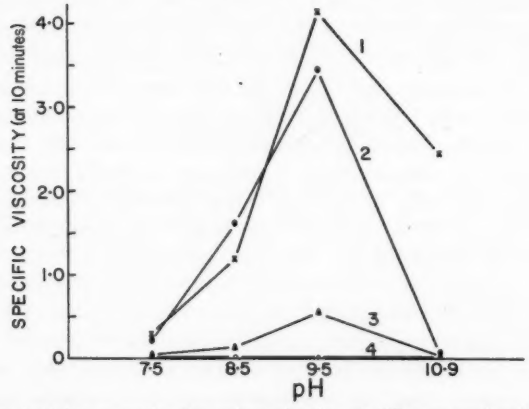

FIG. 1. pH Activity curve for the polymerization of different nucleoside diphosphates by polynucleotide phosphorylase of *A. tumefaciens*. The following buffers were used: pH 7.5 and 8.5, Tris; pH 9.5, glycine–NaOH; pH 10.9, carbonate–bicarbonate. Content of reaction mixture: buffer, 140 μmoles; $MgCl_2$, 5 μmoles; whole extract containing 14 mg protein; nucleoside diphosphate, 25 μmoles (added last at zero time). Total volume: 2.0 ml. Curve 1 = CDP. Curve 2 = ADP. Curve 3 = UDP. Curve 4 = GDP.

ADP and CDP exhibited similar activities with a maximum at pH 9.5. There was only moderate activity toward UDP although the observed peak occurred at the same pH. GDP was not polymerized to a measurable extent at any of the pH's tested. The results obtained with varying Mg^{++} concentrations at pH 9.5 are given in Table I. It can be seen that even in the absence of any added Mg^{++} appreciable activity was obtained with ADP, CDP, and UDP. Since the "whole extract" used for these experiments had not been dialyzed the latter observation can probably be accounted for on the basis of endogenous metal ion content. Maximum activity for ADP and CDP occurred at a molar

TABLE I

Effect of magnesium concentration on polynucleotide phosphorylase activity of an extract of *A. tumefaciens*

$[Mg^{++}]$ / [Substrate]	Specific viscosity at 10 minutes			
	ADP	CDP	UDP	GDP
0	3.10	2.92	1.18	0.00
0.2	3.64	3.46	0.48	0.00
0.3	4.05	—	—	—
0.4	3.64	1.85	0.22	0.00
0.8	1.31	0.59	0.00	0.00

NOTE: Contents of reaction mixture: glycine–NaOH buffer (pH 9.5), 140 μmoles; nucleoside diphosphate, 25 μmoles; $MgCl_2$, varied in order to achieve the ratios to substrate concentration given in the table; whole extract containing 14 mg protein. Total volume: 2.0 ml. The substrate was added at zero time in each case.

Mg^{++}/nucleoside diphosphate ratio of 0.2 to 0.3. The polymerization of UDP seemed to be more efficient under conditions where $MgCl_2$ was not added to the incubation mixture. Again, GDP was inactive at all Mg^{++} concentrations tested.

On the basis of the above results, subsequent experiments with the whole extract were carried out at pH 9.5 and at a Mg^{++}/nucleoside diphosphate ratio of 0.2.

Time Course of Nucleoside Diphosphate Polymerization

In this series of experiments viscosity increase was measured with time in the presence of the individual diphosphates or with two different mixtures of all four diphosphates. The results are given in Table II. With ADP and CDP, specific viscosity increased sharply until a peak was reached at about 20 minutes following the start of incubation. A gradual viscosity decrease was evident thereafter. In agreement with previous results (Fig. 1 and Table I) much lower activity was obtained with UDP with the peak occurring at an earlier time (10 minutes). The observed decrease in specific viscosity is probably due to hydrolysis of the formed polynucleotide by a phosphodiesterase, the presence of which in extracts of *A. tumefaciens*, and action toward poly A, have already been described (3). Polymerization of GDP could not be detected at any time throughout the 30-minute experiment (represented by Table II) nor after 8 hours' incubation. The addition of the mixture of ADP, CDP, UDP, and GDP to the incubation medium at molar concentration ratios of 1:1:1:1 or 1:1:1:0.5, respectively, did not result in any measurable viscosity increase. It may be mentioned at this point that Grunberg-Manago *et al.* (5) synthesized poly AGUC with polynucleotide phosphorylase from *Azotobacter vinelandii* using a concentration ratio of nucleoside diphosphates identical with one of the ratios used for the experiments described here (1:1:1:0.5). The fact that no measurable increase in viscosity could be detected, in the present experiments, indicated that GDP was not only inactive when used alone but that it interfered with the polymerization of the other nucleoside diphosphates.

TABLE II

Time course of polymerization of single and mixed nucleoside diphosphates

Incubation time (minutes)	Specific viscosity					
	ADP	CDP	UDP	GDP	ADP+CDP+UDP+GDP (ratio: 1:1:1:1)	ADP+CDP+UDP+GDP (ratio: 1:1:1:0.5)
5	0.64	0.81	0.79	0.00	0.00	0.00
10	1.50	2.10	1.07	0.00	0.00	0.01
15	1.94	2.81	0.85	0.00	0.00	0.02
20	2.03	2.95	0.62	0.00	0.00	0.02
25	2.00	2.78	0.43	0.00	0.00	—
30	1.81	2.48	0.33	0.00	0.00	0.01

NOTE: Contents of reaction mixture: glycine–NaOH buffer (pH 9.5), 140 μmoles; $MgCl_2$, 5 μmoles; nucleoside diphosphates (singly or in combination per experiment), 25 μmoles (added last at zero time); whole extract containing 14 mg protein. Total volume: 2.0 ml.

Effect of GDP on ADP and CDP Polymerization

A series of experiments was undertaken to study in more detail the inhibition of poly A and poly C synthesis as affected by different concentrations of GDP. The results, presented in Table III, show that (*a*) the addition of 15 μmoles of GDP to the incubation medium, together with 25 μmoles of either ADP or CDP, completely prevented the polymerization of either of the latter substances; (*b*) there was a decreasing degree of inhibition of poly A synthesis with decreasing concentrations of GDP; and (*c*) the same picture of GDP inhibition of ADP polymerization was obtained with an extract of strain A_6 of *A. tumefaciens*. It should be stressed again here that prolonged incubations (up to 8 hours) with 25 μmoles ADP and 15 μmoles GDP did not result in any measurable viscosity increase.

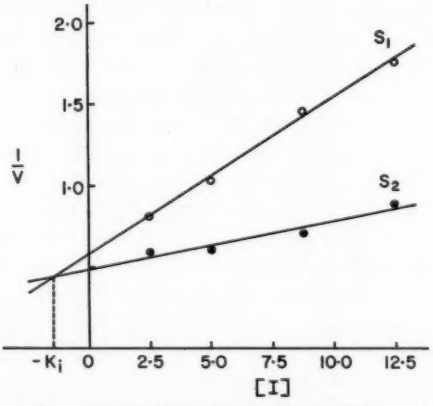

FIG. 2. Lineweaver–Burk–Dixon (12, 13) plot of the inhibition of ADP polymerization by GDP. Contents of reaction mixture: glycine–NaOH buffer (pH 9.5), 140 μmoles; $MgCl_2$, 7.5 μmoles; partially purified enzyme preparation containing 0.35 mg protein; ADP (added last at zero time), 20 μmoles (S_1) or 40 μmoles (S_2). Total volume: 2.0 ml.
[I] = GDP concentration $\times 10^{-4}$ *M*.
$1/V$ = reciprocal of specific viscosity at 10 minutes.

Commercially available GDP could be contaminated by traces of P_i, GTP, GMP, and sometimes even GDP-mannose. (We wish to thank Mr. D. Broida of Sigma Chemical Co. for bringing the latter fact to our attention.) The GDP samples used in our experiments were assayed as 98% pure.* In order to eliminate these small contaminants as possibly contributing to the observed inhibitions by GDP, experiments were set up with quantities of all four substances enumerated above which were at least five times in excess of the maximum contamination, based upon analytical data supplied to us by Sigma Chemical Co. At these concentrations neither P_i, GTP, GMP, nor GDP-mannose resulted in any inhibitory effect in the polymerization system described here.

*Our assay was carried out by electrophoresis, elution, and spectrophotometric analysis.

TABLE III

Effect of guanosine diphosphate on the polymerization of adenosine diphosphate and cytidine diphosphate

Incubation time (minutes)	Specific viscosity									
	Strain B_6							Strain A_6		
	25 μmoles ADP	25 μmoles ADP +15 μmoles GDP	25 μmoles ADP +10 μmoles GDP	25 μmoles ADP +5 μmoles GDP	25 μmoles ADP +1 μmole GDP	25 μmoles CDP	25 μmoles CDP +15 μmoles GDP	25 μmoles ADP	25 μmoles ADP +15 μmoles GDP	ADP:CDP:UDP:GDP (7:7:7:3.5 μmoles)
5	0.71	0	0.04	0.15	0.78	0.81	0	0.75	0	0
10	1.62	0	0.07	0.31	1.49	2.10	0	1.66	0	0
15	1.99	0	0.12	0.47	—	2.81	0	2.13	0	0
20	2.04	0	0.14	0.52	—	2.95	0	2.16	0	0
25	1.93	0	0.16	0.54	—	2.78	0	2.02	0	0

NOTE: Contents of reaction mixture: glycine–NaOH buffer (pH 9.5), 140 μmoles; $MgCl_2$, 5 μmoles; whole extract containing 14 mg protein; nucleoside diphosphates (added last at zero time), as shown. Total volume: 2.0 ml.

In order to investigate the nature of the observed inhibition of poly A formation, experiments were carried out with the 19-fold purified preparation of polynucleotide phosphorylase (see Materials and Methods) at two substrate concentrations and with varying inhibitor concentrations, preparatory to the construction of a Lineweaver–Burk plot (12) as recommended by Dixon (13). The results show (Fig. 2) that GDP inhibits ADP polymerization in a truly competitive manner. The K_i as determined by the above method was found to be 1.5×10^{-4} M.

Discussion

In the present study with the polynucleotide phosphorylase of *A. tumefaciens* it was found that the enzyme exhibits high activity toward ADP and CDP, low activity toward UDP, and none with GDP. Thus, nucleotide specificity seems to be similar to that of the enzyme obtained from *Micrococcus lysodeikticus* and reported by Beers (14) and by Olmsted and Lowe (15, 16). It has been shown also by Grunberg-Manago *et al.* (5) with the *Azotobacter vinelandii* enzyme that reaction with GDP was much slower and required higher enzyme concentrations than with the other nucleoside diphosphates. According to the latter report, however, the authors were able to synthesize poly AGUC at a rate similar to that of homopolymer formation by using a concentration ratio for ADP:GDP:UDP:CDP of 1:0.5:1:1 respectively. In the present study, polymerization could not be observed with this ratio of nucleoside diphosphates at least as far as synthesis of large polymers is concerned. A recent report by Tildon and Szulmajster (17) showed that increasing concentrations of GDP slowed down poly AGUC synthesis, although the same amount of polymer was formed after prolonged incubation (80 hours). The present results show not only that GDP strongly inhibits the polymerization of ADP or CDP but also that this inhibition is competitive.

On the basis of the above considerations it is improbable that polynucleotide phosphorylase is responsible for the biosynthesis of RNA in *A. tumefaciens*. Recent discoveries of new enzymes have, however, provided new alternative pathways. Thus Weiss (18) has described an enzyme system in rat liver which utilizes the ribonucleoside triphosphates for RNA synthesis and is dependent on DNA for activity, and Weiss and Nakamoto (19) have reported a similar system in *Micrococcus lysodeikticus*. Stevens (20) and Hurwitz *et al.* (21) have demonstrated the presence of this enzyme in *Escherichia coli*. In all the above cases it has been suggested that DNA serves as a template since (*a*) the RNA formed is identical in terms of base ratios to the DNA added to the reaction and (*b*) experiments with synthetic deoxyribonucleotide polymers of known base sequence have shown that the base sequence of the synthesized ribonucleotide polymers is precisely directed by the primer employed (22, 23). Recently, Reddi (24) has described an RNA polymerase from spinach leaves which utilizes nucleoside triphosphates but which is dependent on the presence of an RNA–protein complex for activity.

A search for one or more of these enzymes is now in progress with a view to gaining a deeper insight into the true mechanism of RNA biosynthesis in *A. tumefaciens*.

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THE EFFECT OF DIET, AGE, AND SEX ON CHOLESTEROL METABOLISM IN WHITE LEGHORN CHICKENS[1]

J. D. Wood,[2] J. Biely,[3] and J. E. Topliff[2]

Abstract

The cholesterol metabolism of chickens differed in birds of different age and sex. The normal serum cholesterol levels were higher in females than in males and higher in 1-week-old chicks than in mature birds of the same sex. Laying hens were less susceptible to hypercholesterolemia induced by dietary cholesterol than were mature cockerels or young chicks of either sex. Corn oil tended to increase the degree of hypercholesterolemia in all cholesterol-fed birds except young cockerels. A vitamin-A-rich oil, dogfish liver oil, decreased the rise in serum cholesterol level in cholesterol-fed chicks of both sexes, but in mature birds it produced the opposite effect. The addition of 1% cholesterol to the control diet of hens increased the yolk cholesterol concentration of eggs laid by the birds and this increase in concentration was greatly enhanced if 10% corn oil was also present in the diet.

Introduction

Previous work in this laboratory (1) showed that the inclusion of certain fish oils in the diet prevented the onset of hypercholesterolemia induced in young cockerels by cholesterol feeding. The major active factor in the oils was later identified as vitamin A (2). On the other hand, Weitzel *et al.* (3) administered large oral doses of vitamin A to old atherosclerotic hens, and although a marked anti-atherosclerotic effect was observed, the change in the serum cholesterol level was slight. The dissimilar effects of the vitamin could be due to differences in the two investigations which are too numerous to discuss here in detail, but one possibility is that the cholesterol metabolism of chickens is influenced by their age and sex.

That age and sex do have a bearing on serum cholesterol levels of poultry is indicated by the results obtained by several workers. Lorenz *et al.* (4) observed that laying hens had higher blood cholesterol levels than immature hens or mature roosters, and Rodbard *et al.* (5) found that the degree of hypercholesterolemia in cholesterol-fed cockerels depended on the age of the birds. Stamler and co-workers (6) showed that laying hens had to be fed twice as much cholesterol as that given to roosters to obtain the same elevation of serum cholesterol levels.

In view of the above observations the present investigation was initiated to study dietary alteration of serum cholesterol levels in young and old chickens of both sexes. At the same time the effect of the different diets on cholesterol content of eggs was studied.

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Contribution from the Fisheries Research Board of Canada, Technological Station, Vancouver 8, B.C., and the Department of Poultry Science, University of British Columbia, Vancouver 8, B.C.
[2]Fisheries Research Board of Canada, Technological Station, Vancouver 8, B.C.
[3]University of British Columbia, Vancouver, B.C.

The effect of diet on the composition of eggs has been studied by numerous workers. McCollum *et al.* (7), Cruickshank (8), and more recently Reiser (9) and Fisher (10, 11) showed that the composition in egg yolk could be altered by diet but in all cases it was the type of fatty acid in the yolk which was changed. The cholesterol content was shown in the more recent studies to be independent of the amount or type of fat in the hens' diet. The diet used in these investigations did not contain added cholesterol but findings reported by Kurnick *et al.* (12) indicated that even the presence of 1% cholesterol did not alter the cholesterol content of eggs.

Materials and Methods

Birds

White Leghorn birds were used in all experiments. The mature cockerels and laying hens were approximately 8 to 10 months old and the young birds of both sexes were obtained as day-old chicks and kept on the control diet for 7 days prior to the beginning of the tests. The mature roosters were housed in individual cages, the laying hens two to each cage, and the chicks in groups of 16. The older birds were placed in their respective cages 5 days prior to the beginning of the experiment and during this time they were fed the control diet. The studies were carried out during the fall and winter months.

Diets

In order to compare the cholesterol metabolism of birds of different ages and sexes, one should feed the birds the same diet. This is impossible in the case of chickens because of the different dietary requirements of the groups. A control diet for young birds of both sexes and for mature cockerels was formulated as shown in Table I. This diet contained 2% fat and 13% protein. The control

TABLE I

Percentage composition of control diet

Ground wheat	41.20	Distillers' dried solubles	1.87
Ground yellow corn	7.50	Dehydrated cereal grass	0.94
Ground oats	3.75	Iodized salt	0.37
Wheat middlings	3.75	Ground limestone	0.75
Wheat bran	3.75	Manganese sulphate	0.01
Soybean oil meal	5.62	Feeding oil (2250 A–300 D per gram)	0.20
Fishmeal	1.50	Nicarbazin	0.04
Meatmeal	3.75	Sucrose	25.00

Riboflavin 50 mg/100 lb feed

diet for the laying hens had the same composition except that an additional 2% ground limestone and 0.5% bone flour was included to satisfy the laying birds' greater demands for calcium and phosphorus. Additions to the control diets listed in the tables and figures which follow were substituted isocalorically for sucrose, thereby maintaining a constant energy to protein ratio in the diet, a ratio which has been shown (13) to influence serum cholesterol levels in chicks.

Dogfish liver oil from the same batch was used throughout the investigation and its vitamin A content was 8300 I.U./g oil.

The diets were stored at 2° C and fresh feed was placed in the troughs each day to minimize oxidation. The amount supplied to the birds was adjusted so that the calorie intake was approximately the same on all diets. The test diets were continued for 14 days. The duration of the experiments, admittedly rather short, was chosen in view of the results of Rodbard *et al.* (5), which indicated that chicks' susceptibility to cholesterol-induced hypercholesterolemia varied considerably during the first 2 months of their lives. The authors therefore chose a short period (14 days) for the tests, thereby eliminating as far as possible the occurrence of changes in cholesterol metabolism during the test period. Previous experience with chicks indicated that dietary-induced changes in serum cholesterol levels were well established by 14 days.

Determination of Serum Cholesterol

The birds were starved for 18 hours prior to the collection of blood from the brachial vein in order to avoid any short-term changes in serum lipids due to recent ingestion of food by the birds. Blood samples from chicks were pooled in lots of four and the serum collected in the usual manner, but individual samples were used in the case of the mature birds. The total cholesterol concentration in the serum was determined by the method of Sperry and Webb (14).

Determination of Egg Yolk Cholesterol

Estimations were carried out on all eggs individually. The shell was cracked open and the yolk carefully separated from the white of the egg. The yolk was weighed and homogenized for 1 minute with 35–40 ml water using a Servall homogenizer and was then made up to 100 ml with water and mixed thoroughly. The resultant suspension of egg yolk settled slowly but there was ample time to procure aliquots for analysis. Moreover, a brief shaking of a sample which had been standing for several hours produced a suspension which yielded cholesterol analyses which were in close agreement with those obtained using the freshly prepared suspension. An 0.5-ml aliquot from the suspensions was run slowly into a 25-ml volumetric flask containing 10 ml acetone–alcohol (1:1 v/v). The cholesterol determinations were then carried out exactly as described by Sperry and Webb (14) for blood cholesterol.

Identification of Cholesterol and Plant Sterols

The identity of the sterols in corn oil, dogfish liver oil, serum, and egg yolk was determined as follows. Five-gram samples of oil or 10-ml aliquots of egg yolk suspension or serum were saponified with alcoholic alkali and the unsaponifiable material extracted with ether. The ether extract was washed successively with two portions of water, 0.1 *N* KOH, and water until the washings were colorless to phenolphthalein. The ether extract was evaporated to dryness *in vacuo* after most of the solvent had been removed at 60° C under a stream of nitrogen. The residual material was dissolved in ether and made up to a final volume of 10 ml. Aliquots of this solution were chromatographed on Whatman

No. 1 paper impregnated with kerosene, b.p. 220°–240° C, according to the procedure of deZotti *et al.* (15). The mobile phase was pyridine–water (85:15 v/v). The temperature was kept at 20° C instead of the 15° C recommended by de Zotti *et al.* since, unlike the latter workers, we found excellent separation at the higher temperature.

Results

Certain dietary constituents altered the concentration of total cholesterol in the serum of young cockerels (Table II). The chicks were highly susceptible to cholesterol-induced hypercholesterolemia, the serum cholesterol being increased sixfold when 1% cholesterol was included in the diet. The presence of corn oil did not alter this effect but dogfish liver oil, in agreement with previous findings, reduced the degree of hypercholesterolemia. It should be mentioned at this point that all differences in serum cholesterol level reported in this paper are statistically significant at $P = 0.01$ unless otherwise stated.

TABLE II

The effect of diet on serum cholesterol levels in 7-day-old cockerels

Diet	Food consumption (g/bird/day)	Relative calorie intake	Weight gain (g)	Serum cholesterol (mg/100 ml)
Control	16.0	100	68.1	183 ± 5*
Control + 1% cholesterol	15.4	103	63.6	1099 ± 281
Control + 1% cholesterol + 10% corn oil	14.6	106	76.0	1028 ± 152
Control + 1% cholesterol + 10% dogfish liver oil	14.4	104	56.3	435 ± 86

*Mean value for four groups of serum ± the standard deviation. Each group contains serum from four birds.

The mean weight gain of the cockerels varied considerably with the type of diet in spite of the fact that the calorie intake was approximately the same on all diets. In particular, the weight gain of birds of the fish oil diet was low. Similar low weight gains were observed in most experiments conducted in these laboratories with various fish oils possessing hypocholesterolemic activity, and these findings might suggest that the oils were perhaps toxic to growing chicks and that the associated lower cholesterol levels were a non-specific resultant thereof. However, in some experiments the rate of growth of chicks on fish-oil-supplemented diets was comparable to that of birds on the cholesterol-containing control diets and significant hypocholesterolemic activity was still in evidence (Table III). It would appear therefore that there is no definite association between the hypocholesterolemic effect and the depression in growth rate.

In young pullets the dietary regimens produced effects similar to, but not identical with, those observed in the male chicks (Table IV). The serum cholesterol level in the "control" birds was higher than in young cockerels and the hypercholesterolemic effect of 1% cholesterol was increased by the presence

TABLE III

Hypocholesterolemic activity of fish-oil-supplemented diets which support a growth rate in chicks similar to that observed with cholesterol-containing control diets

Expt. No.	Duration of experiment (days)	Addition to control diet containing 1% cholesterol	Food consumed (g/chick/day)	Weight gain (g)	Serum cholesterol (mg/100 ml)
1	10	None	16.0	48.3	686 ± 220*
		2% lingcod liver oil (42,500 I.U. vit. A/g)	13.6	46.6	476 ± 65
2	14	None	13.7	63.6	990 ± 68
		9% lingcod liver oil (18,900 I.U. vit. A/g)	14.2	65.6	179 ± 23
3	14	None	14.5	64.7	983 ± 214
		10% dogfish liver oil (8,000 I.U. vit. A/g)	13.8	63.1	556 ± 62

*Mean value for four groups of serum ± the standard deviation. Each group contains serum from four birds.

of 10% corn oil, although, due to the large variation in the individual values, this "additional" increase was not significant at $P = 0.01$. Dietary dogfish liver oil acted as a hypocholesterolemic agent and also reduced the rate of growth of the chicks, as was the case with cockerels.

TABLE IV

The effect of diet on serum cholesterol levels in 7-day-old-pullets

Diet	Food consumption (g/bird/day)	Relative calorie intake	Weight gain (g)	Serum cholesterol (mg/100 ml)
Control	17.0	100	56.2	248 ± 7*
Control + 1% cholesterol	16.6	99	58.0	847 ± 120
Control + 1% cholesterol + 10% corn oil	14.0	96	59.1	1092 ± 262
Control + 1% cholesterol + 10% dogfish liver oil	13.3	91	38.6	491 ± 144

*Mean value for four groups of serum ± the standard deviation. Each group contains serum from four birds.

The effect of diet on serum cholesterol levels in mature cockerels is shown in Table V. The cholesterol level of birds on the control diet was lower than that observed with young birds of either sex. The incorporation of 1% cholesterol

TABLE V

The effect of diet on serum cholesterol levels in mature cockerels

Diet	Food consumption (g/bird/day)	Relative calorie intake	Mean change in body weight (lb)	Serum cholesterol (mg/100 ml)
Control	122.6	100	−0.1	134 ± 19*
Control + 1% cholesterol	123.9	103	−0.1	599 ± 328
Control + 1% cholesterol + 10% corn oil	104.4	101	0.0	707 ± 312
Control + 1% cholesterol + 10% dogfish liver oil	90.8	86	−0.1	980 ± 366

*Mean value for 12 birds ± the standard deviation.

into the diet caused a 4.5-fold increase in serum cholesterol concentration. This elevated cholesterol level was increased still further by both corn oil and dogfish liver oil, but only with the latter oil was the increase, over and above that caused by the dietary cholesterol, significant. The body weight of the mature cockerels did not alter appreciably during the course of the experiment.

The cholesterol levels of laying hens on the test diets are shown in Table VI. The level in the control birds was higher than in mature cockerels but lower than in young pullets. Dietary cholesterol itself failed to increase significantly

TABLE VI

The effect of diet on serum cholesterol levels in laying hens

Diet	Food consumption (g/bird/day)	Relative calorie intake	Mean change in body weight (lb)	Serum cholesterol (mg/100 ml)
Control	116.2	100	0.0	177 ± 109*
Control ± 1% cholesterol	118.9	104	−0.1	203 ± 85
Control + 1% cholesterol + 10% corn oil	109.4	109	0.0	295 ± 143
Control + 1% cholesterol + 10% dogfish liver oil	101.2	101	−0.1	593 ± 291

*Mean values for 16 birds ± the standard deviation.

the serum cholesterol concentration, but in conjunction with corn oil the sterol did produce a significant elevation. Dogfish liver oil was still more effective in this respect than corn oil. As was the case with the mature cockerels the body weight of the laying hens did not alter appreciably during the period of investigation.

The egg production by the hens was influenced by dietary constituents as can be seen from Fig. 1. The incorporation of 1% cholesterol with or without 10% corn oil into the control diet brought about little change in the rate of egg production, but the presence of 10% dogfish liver oil in the cholesterol-containing diet caused a noticeable decrease in production around 5 days and a complete cessation of laying after 7 days on the diet.

Although there was no striking difference in the number of eggs laid by hens on the first three diets mentioned above, the composition of the egg yolks varied considerably (Fig. 2). The incorporation of 1% cholesterol in the diet increased the concentration of egg yolk cholesterol and this effect was enhanced by the presence of 10% corn oil, the value after the 14 days experimental period being twice that in the control eggs. A statistical analysis was performed on the data and Table VII gives the significance obtained for the differences in yolk cholesterol concentration between the diets on individual days. A significant increase in yolk cholesterol of eggs from birds on the cholesterol-containing diet became manifest after 7 days, but this increase was significant after only 5 days when corn oil was also present in the diet. Figure 2 and Table VII do not show the data obtained for eggs from hens fed the dogfish liver oil diet, but during the

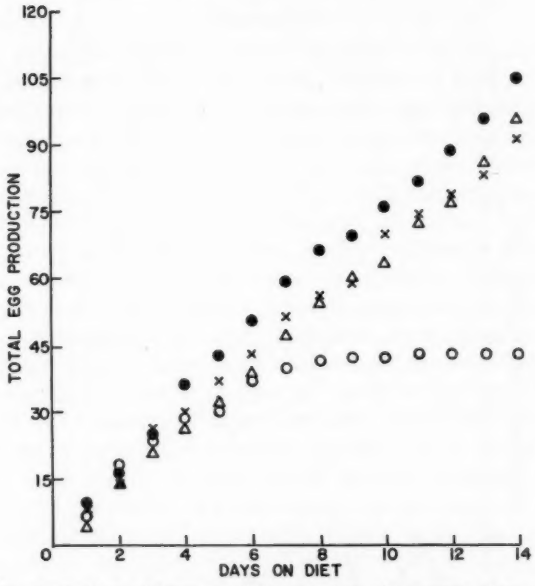

FIG. 1. Egg production of hens on different diets. ● control diet; △ control diet +1% cholesterol; × control diet + 1% cholesterol + 10% corn oil; ○ control diet + 1% cholesterol + 10% dogfish liver oil.

TABLE VII

Significance of differences between egg yolk cholesterol concentrations

Days on diet	Level of probability at which difference is significant		
	Diets 1 and 2* (%)	Diets 1 and 3 (%)	Diets 2 and 3 (%)
1	N.S.†	N.S.	N.S.
2	N.S.	N.S.	N.S.
3	N.S.	N.S.	N.S.
4	N.S.	N.S.	N.S.
5	N.S.	1	N.S.
6	N.S.	5	N.S.
7	1	1	5
8	1	1	5
9	5	1	5
10	1	1	1
11	1	1	1
12	1	1	1
13	1	1	1
14	1	1	1

*Diets 1, 2, and 3 are the control diet, control diet + 1% cholesterol, and control diet + 1% cholesterol + 10% corn oil, respectively.
†N.S. denotes difference is not significant.

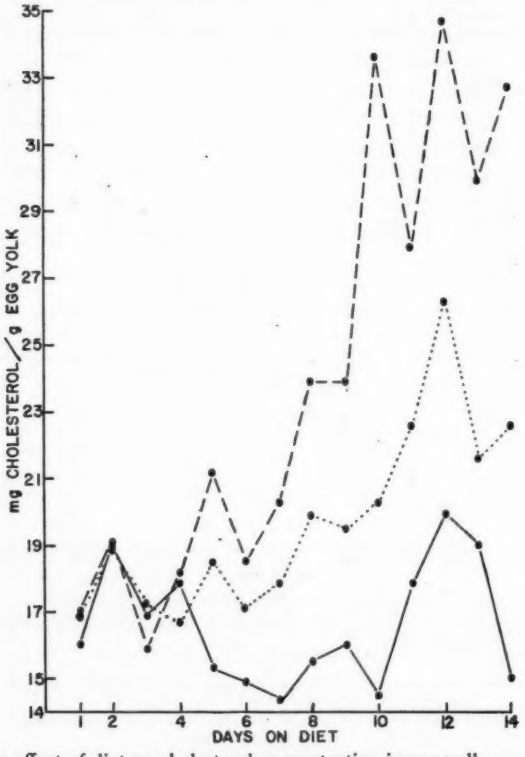

FIG. 2. The effect of diet on cholesterol concentration in egg yolk. —— control diet; . . . control diet + 1% cholesterol; – – – control diet + 1% cholesterol + 10% corn oil.

few days that the birds on this diet laid eggs the egg yolk cholesterol concentration was similar to that in eggs from the control hens. The above results deal with the concentration of cholesterol in the yolks but since the weights of the latter were similar on all diets, the same picture is obtained for the total amount of cholesterol in the eggs.

The unsaponifiable fractions of corn oil and dogfish liver oil were compared with cholesterol and β-sitosterol standards using the chromatographic system described earlier in this paper. Corn oil gave a spot with the same R_f as β-sitosterol and the fish liver oil gave a spot corresponding to cholesterol. No other spots were observed with the material from the oils. Similar studies with the unsaponifiable fractions from the sera of laying hens on the four diets gave in all cases one spot which corresponded to cholesterol. The unsaponifiable material from the yolks of eggs laid on the 14th day of the experiment was examined chromatographically and the results indicated that, regardless of the diet, egg yolks contained cholesterol with no observable contamination of plant sterols.

Discussion

Age and sex appeared to have a strong bearing on the mechanism of cholesterol metabolism in chickens. The normal cholesterol level in young chicks was higher than that in mature birds of the same sex, this decrease with age occurring in both sexes. Moreover, in both young and old birds, males had lower serum cholesterol concentrations than females, an observation which agrees with the work of Lorenz *et al.* (4) which indicated that laying hens had somewhat higher blood total cholesterol values than mature cockerels.

The susceptibility of chickens to hypercholesterolemia induced by dietary cholesterol also varied with the age and sex of the birds. The addition of 1% cholesterol to the control diet brought about an approximately four- to six-fold increase in the serum cholesterol concentration of young pullets and of cockerels of both age groups. On the other hand, the laying hens were more resistant to this type of hypercholesterolemia under the conditions of the present experiment. Hens fed the cholesterol-containing diet for 2 weeks showed no significant increase in the serum cholesterol level but it might well be that a longer period would produce a significant increase. When the diet contained 10% corn oil and 1% cholesterol the serum cholesterol level of the hens increased, but the degree of hypercholesterolemia obtained was still much less than that which occurred in cockerels and young pullets. Since the protein content of the diets was constant, rapidly growing chicks were perhaps suffering a protein lack relative to the situation in laying hens of constant body weight, but it is unlikely that this relative lack was a major cause of the difference in susceptibility to hypercholesterolemia of young chicks and laying hens because mature cockerels of constant body weight were also readily susceptible to dietary-induced cholesterolemia.

The possibility that the presence of plant sterols from ingested corn oil contributed significantly to the observed elevation of serum sterol concentration in corn-oil-fed hens was rendered improbable by the results of the chromatographic analysis which indicated no large amount of plant sterols in the serum. This observation is analogous to the finding of Swell *et al.* (16) that the ingestion of plant sterols by rats did not lead to an accumulation of the sterols in the serum.

The action of dogfish liver oil on cholesterol-induced hypercholesterolemia depended on the age of the birds but not on their sex. In young birds the liver oil reduced the degree of hypercholesterolemia by approximately 50% but complete prevention could be effected by increasing the amount of dietary fish oil (Wood and Topliff (2)). In contrast to this behavior, the addition of dogfish liver oil to the cholesterol-containing diet increased the degree of hypercholesterolemia in mature birds. Since the hens stopped laying when the fish oil was present in the diet, it was thought that perhaps the observed increase in serum cholesterol concentration was related to this phenomenon, but such an explanation does not account for the similar action of the oil in mature cockerels. A possible reason for the elevation of serum cholesterol is the presence of cholesterol in dogfish liver oil. The oil used in the experiments con-

tained 1.4% sterol which was shown by chromatographic analysis to be mostly, if not entirely, cholesterol. The amount of "fish oil cholesterol" therefore amounted to 0.14% of the diet. Whether the increase in the amount of dietary cholesterol from 1.00% to 1.14% caused the marked elevation in cholesterol level is open to question. Another possible explanation is that glyceryl ethers present in dogfish liver oil (17) increased the intestinal absorption of cholesterol by reason of their emulsifying properties (18).

Earlier work in this laboratory (2) indicated that the hypocholesterolemic action of fish oils was due in most part to their vitamin A content, and it must be inferred, therefore, from the present results that vitamin A does not possess this action in mature birds. This agrees with the report of Weitzel *et al.* (3) which indicated that vitamin A did not lower serum cholesterol levels in old hens. The vitamin may, however, be responsible for the cessation of egg production observed in the present investigation when dogfish liver oil was incorporated into the diet, since excessive amounts of vitamin A have been shown to affect adversely the reproductive ability of the rat (19) and the mink (20).

The amount of cholesterol in egg yolk was profoundly influenced by the diet of the hens. The presence of 1% cholesterol in the diet increased the egg yolk cholesterol content, an elevation which was enhanced by the incorporation of 10% corn oil into the feed. This result is contrary to the work of Kurnick *et al.* (12), who reported that dietary cholesterol produced no change in egg cholesterol. The difference in the observations may be due to a difference in composition of the experimental diets, especially since the present investigation has shown that the fat content of the cholesterol-containing diet played an important role in the control of egg yolk cholesterol. The experiments reported here were of short duration and longer-term investigations are planned for the near future to ascertain whether the changes in egg yolk cholesterol continue over an extended period.

It is clear from the above results and discussion that laying hens were more resistant to cholesterol-induced hypercholesterolemia than were the other birds, no matter whether the cholesterol was incorporated into the diet alone or together with corn oil. This finding agrees with that of Stamler *et al.* (6) which indicated that a higher dietary cholesterol level was required by laying hens than by roosters to produce the same degree of hypercholesterolemia. One possible explanation is that the hens can eliminate cholesterol via the eggs, a route not available to the other birds. Evidence for this mechanism was brought forth by Stamler's group (6), who showed that the greater resistance of hens to dietary-induced hypercholesterolemia was abolished by oviduct ligation in the birds. The present investigation furnishes further proof by showing that dietary components which caused hypercholesterolemia in chickens also increased the cholesterol content of eggs laid by the hens. If the above proposed mechanism is correct, then substances which cause a cessation of egg production

in cholesterol-fed hens should elevate the serum cholesterol levels. This situation arose in the present studies when 10% dogfish liver oil was included in the diet. The hens stopped laying within 7 days and, in agreement with the proposed theory, a substantial increase in serum cholesterol concentration occurred. The concentration was still lower than that in cockerels fed a comparable diet but this was not unexpected since the hens laid a considerable number of eggs in the first half of the test period and this would doubtless influence the cholesterol level reached after 14 days. However, as pointed out previously, other complicating factors were involved in the action of the dogfish liver oil. The above explanation for the greater resistance of laying hens to hypercholesterolemia cannot be ignored but it is unlikely to be the sole cause since an estrogenic effect on the degree of lipemia and cholesterolemia in chickens has previously been reported (21, 22).

The work reported here has indicated that dietary constituents which increase serum cholesterol levels in hens also increase the cholesterol content of the eggs laid by the birds. It would be interesting if the converse were true and hypocholesterolemic agents decreased egg cholesterol levels, especially since the current trend is to cut down on the human consumption of cholesterol-rich foods.

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COMPETITION BETWEEN AMINO ACIDS FOR TRANSPORT INTO EHRLICH ASCITES CARCINOMA CELLS[1]

P. G. SCHOLEFIELD[2]

Abstract

The cumulative entry of amino acids into Ehrlich ascites carcinoma cells is due to the presence of active transport systems, each with its own specific range of substrates. Several amino acids and amino acid analogues may have an affinity for the same transport system and thus may inhibit transport of other amino acids by acting as competitive inhibitors or competitive substrates. Loss of methionine from ascites cells takes place by a diffusion process which obeys Fick's law. Leucine accumulation by ascites cells is small and is increased on addition of certain other amino acids. The increase is not due to inhibition of leucine oxidation as increase in the rate of production of radioactive carbon dioxide from labeled leucine also occurs. Kinetic aspects of these results are discussed.

When Ehrlich ascites carcinoma cells are incubated in a medium containing amino acids, it is found that the cells accumulate the amino acids (1). The extent of the accumulation is such that when glycine is present in the incubation medium at a concentration of 2 mmolar, the steady-state level inside the cells often exceeds 20 mmolar. The above experiments of Christensen and Riggs (1) also provide evidence that simultaneous production of energy is necessary for such accumulation of amino acids and that a biological transport system is involved (see also ref. 2). The kinetics of the transport process were studied by Heinz (2), who showed that the progress of the amino acid transport can be expressed in terms of the equation

$$U = U_\infty(1-e^{-kt}), \qquad [1]$$

where U represents the uptake, U_∞ the maximal uptake, k is a constant, and t is the time. It has also been reported by Heinz (2) that the steady-state levels of amino acids attained in the experiments of Christensen and Riggs (1) can be correlated with the extracellular concentration of amino acid (glycine) by the relation

$$v^0_{max}\frac{a_i}{K_m+a_i} = k_{diff}(a_e-a_i), \qquad [2]$$

where v^0_{max} is the maximum initial velocity of active transport, a_e and a_i are the intracellular and extracellular glycine concentrations respectively, K_m is a Michaelis-type constant, and K_{diff} the diffusion constant across the cell boundary.

Cohen and Monod (3) employed a similar equation to express their results on thiogalactoside accumulation in *Escherichia coli* but neglected the a_i term

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Contribution from the McGill–Montreal General Hospital Research Institute, 3619 University Street, Montreal, Que.
[2]National Cancer Institute of Canada, Associate Professor of Biochemistry, McGill University, Montreal, Que.

on the right-hand side of equation [2]. Since the thiogalactosides may be concentrated, in some instances, to the extent of more than 100-fold, this simplification seems to be warranted. The simplified equation has been applied to amino acid transport into Ehrlich ascites cells by Tenenhouse and Quastel (4) and Johnstone and Scholefield (5), and the application appears to be valid for glycine (4, 5), serine (4), and proline (4). The simplified equation does not appear to describe completely the kinetics of S-ethylcysteine transport as investigated by Tenenhouse and Quastel (4). However, a personal communication from these authors suggests that the S^{35}-labeled L-amino acid may have contained an appreciable quantity of the D-isomer. A preliminary application of the Lineweaver and Burk (6) formulation of competitive inhibition to methionine inhibition of glycine transport gave the predicted straight-line relationship only at low concentrations of methionine (5).

Interaction among amino acids has been studied many times in connection with transport into Ehrlich ascites cells. Riggs, Coyne, and Christensen (7) showed that glycine may displace previously concentrated L-diaminobutyric acid and that L-diaminobutyric acid may displace previously concentrated glycine. Jacquez (8) has suggested that the interaction may be of a competitive nature since incubation of Ehrlich ascites cells with glycine, glutamine, or tryptophan, but not with glutamic acid, partially prevents the uptake of azaserine. The competitive effects of other amino acids on the transport and accumulation of S-ethylcysteine have also been investigated (4), and it has been suggested that several transport systems are involved in the accumulation of the various amino acids (4).

The objectives of the present work were therefore (i) to investigate the applicability of the laws of enzyme kinetics to amino acid transport in Ehrlich ascites cells, and (ii) to determine if the classical equations used in defining the nature of enzyme inhibition through studies of enzyme kinetics may safely be applied to interaction among amino acids during transport into Ehrlich ascites cells.

Materials and Methods

Animals

All mice used were male CF1 Swiss white mice weighing 20–25 g purchased from Carworth Farms, New York, U.S.A.

Ascites Cells

The Ehrlich ascites cells were harvested 6 or 7 days after intraperitoneal injection of 0.4 ml ascitic fluid. The yield of cells and the metabolic activity were maintained at a high and constant level only if the ascitic fluid used as inoculum had been derived from the solid form of the tumor (Ehrlich carcinoma). This form could easily be maintained by subcutaneous injection of ascites cells so that the usual cycle for the cells used in the present work was solid to liquid (used as inoculum) to liquid (harvested, or used for production of solid tumors). The ascites cells were washed in ice-cold saline to remove

blood elements, packed, and resuspended in Ringer solution as previously described (5).

Incubation Technique

The incubation medium was a calcium-free Krebs–Ringer phosphate solution of the following composition: 145 mmolar NaCl, 5.8 mmolar KCl, 1.5 mmolar KH_2PO_4, 1.5 mmolar $MgSO_4$, and 10 mmolar phosphate buffer (pH 7.4). Labeled and non-labeled amino acids were added as indicated. The reaction was begun by addition of 1 ml of a 1:12 dilution of ascites cells to the incubation medium in 25 ml Erlenmeyer flasks. The total volume was 3 ml and, unless otherwise stated, the incubation was carried out for 45 minutes at 37°. At the end of this period, steady-state levels of radioactive amino acids had been attained within the cells (5, 8, 13).

Alcohol-Soluble Amino Acids

The reaction was terminated by removing the flasks from the incubation bath and placing them on crushed ice. To each flask was added 8 ml ice-cold, calcium-free Krebs–Ringer solution; the mixture was shaken and transferred to centrifuge tubes. The cells were sedimented by centrifuging for 1 minute at 800 g, the supernatants decanted, and any excess fluid removed with tissue paper. Further similar washings had little effect on the levels of alcohol-soluble amino acids (see also ref. 4). Amino acids were extracted from the pellet by addition of 3 ml 95% ethanol. The mixture was allowed to stand with occasional stirring for 30 minutes to complete the extraction, centrifuged, and an aliquot (300 μl) removed for plating and counting.

Alcohol-Insoluble Amino Acids

In some experiments the effects of other amino acids on the incorporation of glycine and methionine into the alcohol-insoluble fraction were investigated. These were performed as previously described (5), the figures obtained giving a reasonable indication of the extent of incorporation of radioactive amino acid into protein.

Radioactive Amino Acids

Glycine-1-C^{14}, DL-methionine-S^{35}, and DL-leucine-1-C^{14} were obtained from Merck and Co. Ltd., Montreal.

Amino Acid Analogues

All the amino acid analogues used in the present work were DL-mixtures and were kindly donated by Dr. L. Berlinguet of Laval University.

Counting Techniques

A Geiger–Müller thin end-window tube and Baird Atomic "Abacus" 123 Scaler were used. No corrections for self-absorption were necessary except when C^{14}-labeled barium carbonate was counted. In this case the activity was corrected to infinite thinness according to the method of Calvin, Heidelberger, Reid, Tolbert, and Yankwich (9). All samples were counted for a sufficient length of time to ensure an accuracy of at least $\pm 3\%$.

Theoretical

Rearrangement of equation [2] gives

$$\frac{v^0_{max}/k_{diff}}{a_e - a_f} = 1 + \frac{K_m}{a_f},$$

which may be simplified to

$$[3] \qquad \frac{A}{a_e - a_f} = 1 + \frac{K_m}{a_f},$$

where $A = v^0_{max}/k_{diff}$.

If the cellular concentration in the steady state is great compared with the concentration in the medium, i.e. if a_e is much greater than a_f, then equation [3] simplifies to

$$[4] \qquad \frac{A}{a_e} = 1 + \frac{K_m}{a_f}.$$

When a_f becomes very large, equation [3] simplifies to

$$A = a_e - a_f$$

so that A represents the maximum concentration gradient which may be maintained across the cell membrane and is determined by the ratio of the maximum initial velocity of transport to the diffusion constant (v^0_{max}/k_{diff}).

The concentration of amino acid in the medium a_f is most conveniently expressed as μmoles/ml or mmolar. Analysis of the amount of radioactive amino acid present in the cells permits a_e to be calculated as μmoles/ml packed cells and a factor is required to calculate the amount of intracellular fluid to which glycine may have access in each ml packed cells. The trapped extracellular fluid in well-packed ascites cells has been estimated by Heinz and Mariani (10) to be 0.16 ml/g packed cells or 0.16 ml/ml packed cells. The dry weight of 1 ml packed cells, prepared as described in Materials and Methods, is 90–100 mg (unpublished measurements by Dr. R. M. Johnstone of this Institute). The remaining material (0.74–0.75 g/ml packed cells) was presumed to be a measure of the intracellular water and a preliminary figure of 0.75 was therefore used in the present calculations. Substitution of other factors such as 0.70 or 0.80 led to results which were not linear when plotted by the method of Lineweaver and Burk (6).

Results

(i) Glycine-1-C^{14}

The effects of several amino acid analogues on the steady-state level of glycine-1-C^{14} in Ehrlich ascites cells and on the incorporation of glycine-1-C^{14} into the alcohol-insoluble fraction of these cells may be seen from the values quoted in Table I. None of the analogues studied is a particularly good inhibitor of glycine incorporation although all of them, with the exception of 1-aminocyclohexane carboxylic acid, have some effect on the uptake of glycine.

The related compound, 1-aminocyclopentane carboxylic acid, is the most effective of the analogues examined.

TABLE I

The effects of several amino acid analogues on glycine-1-C^{14} uptake into the alcohol-soluble fraction and its incorporation into the alcohol-insoluble fraction of Ehrlich ascites cells

Experiment	Analogue added	Concentration (mmolar)	Uptake*	Incorporation†
1	Nil	—	10.7	0.24
		—	10.6	0.26
	1-Aminocyclopentane carboxylic acid	2	6.7	0.21
		4	4.6	0.22
	1-Aminocyclohexane carboxylic acid	2	10.4	0.25
		4	10.2	0.27
	N-Methyl-2-phenyl glycine	2	9.1	0.24
		4	8.1	0.23
	Allyl glycine	2	7.7	0.26
		4	6.6	0.25
	3-Amino-3-carboxypropane sulphonamide	2	8.3	0.23
		4	6.8	0.21
2	Nil	—	14.4	0.39
	Allyl glycine	3	10.1	0.42
		6	8.2	0.34
	Furyl glycine	2	11.7	0.36
		6	10.4	0.34
	Thienyl glycine	2	12.7	0.41
		4	11.6	0.44

NOTE: Ehrlich ascites cells were incubated with 2 mmolar glycine-1-C^{14} for 45 minutes at 37° C and the various fractions isolated, plated, and counted as described in Materials and Methods.
*μmoles glycine taken up into the alcohol-soluble fraction/ml packed cells.
†μmoles glycine incorporated into the alcohol-insoluble fraction/ml packed cells.

On the basis of these results, the kinetics of the inhibitions of amino acid transport produced by some of the analogues were examined in detail.

(a) 1-Aminocyclopentane Carboxylic Acid

In preliminary experiments the concentrations of radioactive glycine (substrate) and amino acid analogue (inhibitor) were varied independently. The Lineweaver and Burk formulation for competitive inhibition was included in equations [3] and [4], and applied to the observed inhibitory effects. Kinetic analysis of the results indicated that the inhibition is competitive whichever equation is used. Subsequent experiments were designed to yield the greatest accuracy in the determination of the characteristic constants rather than to give repeated confirmation of the competitive nature of the inhibition. Approximately one-half of the flasks were used to estimate glycine uptake at various substrate concentrations (with no inhibitors present), and the other half to estimate the effects of various concentrations of inhibitor at one substrate concentration. The results were then plotted by the method of Lineweaver and Burk (6) ($1/a_e$ or $1/a_e - a_f$ against $1/a_f$) or by the method of Dixon (11) ($1/a_e$ or $1/a_e - a_f$ against 1/inhibitor concentration). A set of values obtained in a typical experiment of this type is quoted in Table II. The figures

TABLE II

A comparison of the theoretical and observed levels of radioactivity in aliquots of the alcohol-soluble fraction of ascites cells after attainment of steady-state levels of glycine-1-C^{14} in the presence of various amounts of this substrate and of 1-aminocyclopentane carboxylic acid

Vessel number	Glycine-1-C^{14} (mmolar)	1-Amino-cyclopentane carboxylic acid (mmolar)	c.p.m./aliquot		
			Calculated* from equation [5]	Observed	Calculated† from simplified form of equation [5]
1	8.0	0	4082	4036	4036
2	6.0	0	2646	2758, 2540	2691
3	2.6	0	1941	1856	1980
4	2.0	0	1583	1566	1614
5	1.6	0	1320	1334	1345
6	1.3	0	1116	1063	1130
7	1.0	0	883	891, 805	897
8	2.0	10.0	452	425	375
9	2.0	8.0	496	444	444
10	2.0	6.4	546	526	520
11	2.0	4.8	620	604	626
12	2.0	3.2	739	779	787
13	2.0	1.6	970	1016	1060

NOTE: Incubation, isolation, and counting were carried out as described in Materials and Methods. Specific activity of glycine 18.63 c.p.m./mμmole.

*Calculated assuming K_m = 6.4 mmolar, K_i = 1.47 mmolar, and A = 5670 c.p.m./aliquot. A is a measure, in this case, of the maximum concentration gradient.

†Calculated assuming K_m = 8.4 mmolar, K_i = 2.43 mmolar, and A = 8072 c.p.m./aliquot. A represents, in this case, the maximum of alcohol-soluble radioactivity (mainly glycine-1-C^{14}) that the cells can retain.

corresponding to vessels 1–7 refer to counts per minute per aliquot of the alcohol-soluble fraction after the cells had attained a steady state and are a measure of the intracellular glycine concentration, a_c. For use in the original equation, these figures must be reduced to correspond to the concentration gradient rather than to the concentration of glycine in the cell, i.e. to correspond to $a_c - a_f$ rather than to a_c. For example, in vessel 1 the observed value is 4036 c.p.m. when the original extracellular concentration of glycine is 8 mmolar or 8 μmoles/ml, and the specific activity of the glycine-1-C^{14} present is 18.63×10^3 c.p.m./μmole. The figure to be subtracted is, therefore, 18.63×10^3 multiplied by 8 (to give c.p.m./ml), divided by 12 (since only 1/12 ml packed cells was used per vessel), divided by 10 (since an aliquot of 300 μl was taken from the 3 ml ethanol used for extraction), multiplied by 0.75 (to refer values from ml packed cells to ml fluid/ml packed cells) or

$$\frac{18.63 \times 10^3 \times 8 \times 0.75}{12 \times 10} = 932.$$

The value used in place of 4036 was, therefore, 4036 − 932 = 3104 c.p.m.

The reciprocals of the corrected $(a_c - a_f)$ and of the uncorrected (a_c) values are shown in Fig. 1, plotted against the reciprocal of the concentration of glycine in the medium. Both sets of values appear to obey a linear relationship. From each of the lines so obtained, values for A and K_m were calculated.

The figures corresponding to vessels 8–14 in Table II refer similarly to aliquots of the alcohol-soluble fraction, but the concentration of glycine-1-C^{14}

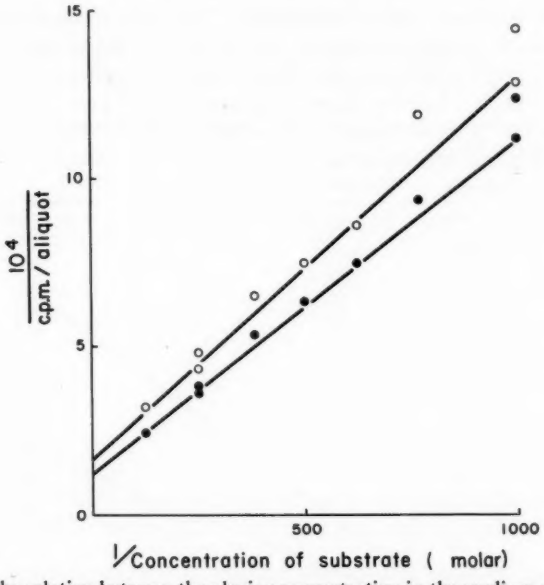

FIG. 1. The relation between the glycine concentration in the medium and the radioactivity per aliquot of the alcohol-soluble fraction of ascites cells after incubation with glycine-1-C^{14} (specific activity, 18.63 c.p.m./mμmole): ●, no correction applied; ○, correction applied as indicated in the text.
Incubation was carried out as described in Materials and Methods.

was kept at 2 mmolar throughout, and the concentration of 1-aminocyclopentane carboxylic acid was varied from 1.6 to 10 mmolar. Corrected values were obtained and the reciprocals, together with those of the uncorrected values, are shown, plotted against the concentration of inhibitor in the medium, in Fig. 2. Both sets of values appear to approximate equally well to a straight line at the lower concentrations of inhibitor and, from the two gradients, values for K_i were calculated from the equation

$$[5] \qquad \frac{A}{a_e - a_f} = 1 + \frac{K_m}{a_f}\left(1 + \frac{[I]}{K_i}\right),$$

where [I] is the concentration of inhibitor and K_i is the Michaelis-type constant for this inhibitor. In the simplified version of this equation a_e again replaces $a_e - a_f$ in the term on the left-hand side.

From the values found for A, K_m, and K_i, the expected values for the levels of radioactivity in the aliquots were calculated for different values of a_f and [I]. These are the theoretical values quoted in Table II. It is of interest to note that the theoretical maximum concentration gradient (A) calculated from equation [5] is equivalent to 5670 c.p.m./aliquot or

$$\frac{5670 \times 12 \times 10}{18.63 \times 10^3 \times 0.75} \text{ mmolar} = 49 \text{ mmolar}$$

(for the method of calculation, see above). The value determined experimentally by Heinz and Mariani (10) is 45 ± 14 mmolar. It should be noted, however, that Heinz (2) and Tenenhouse and Quastel (4) are in agreement that the value of K_m is 3.7 mmolar, but the present values are 6.4 mmolar and 8.4 mmolar depending on the equation used. The value quoted by Heinz (2) could be in error since it is dependent on estimation of levels of radioactivity after incubation for only 2 minutes.

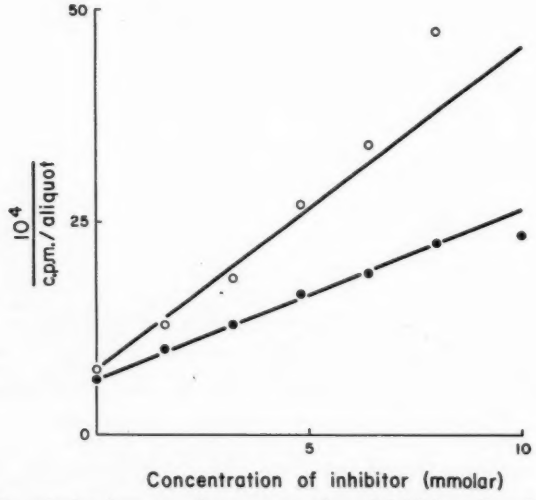

FIG. 2. The relation between the concentration of inhibitor (1-aminocyclopentane carboxylic acid) and the reciprocal of the radioactivity per aliquot of the alcohol-soluble fraction of ascites cells after incubation with glycine-1-C^{14} (specific activity, 18.63 c.p.m./ mμmole): ●, no correction applied; ○, correction applied as indicated in the text. Incubation was carried out as described in Materials and Methods.

(b) Allyl Glycine and 3-Amino-3-carboxypropane Sulphonamide

The results obtained with these two inhibitors are presented in Tables III and IV. The two approaches again lead to predicted values which approximate the observed results equally well. In each case the Michaelis constant for glycine and the maximum concentration gradient or the maximum uptake of glycine are in good agreement with the corresponding values quoted in Table II.

(ii) DL-*Methionine-*S^{35}

The results obtained with glycine-1-C^{14} show that the simplified version of equation [5] fits the experimental results as well as the original equation [5]. However, the values calculated for the constants A, K_m, and K_i and the significance to be attached to the first constant (A) are quite different in the two approaches. It was felt that use of DL-methionine, which is not as well concentrated as glycine (12, 13), might yield less equivocal results.

TABLE III

A comparison of the theoretical and observed levels of radioactivity in aliquots of the alcohol-soluble fraction of ascites cells after attainment of steady-state levels of glycine-1-C^{14} in the presence of various amounts of this substrate and of allyl glycine

Vessel number	Glycine-1-C^{14} (mmolar)	Allyl glycine (mmolar)	c.p.m./aliquot		
			Calculated* from equation [5]	Observed	Calculated† from simplified form of equation [5]
1	6.0	0	3642	3641	3544
2	3.0	0	2281	2209	2255
3	2.0	0	1670	1674	1654
4	1.6	0	1392	1394	1378
5	1.3	0	1170	1182	1154
6	1.0	0	930	896	919
7	2.0	3.2	902	902	897
8	2.0	2.4	1004	1046	1014
9	2.0	1.8	1106	1110	1123
10	2.0	1.2	1237	1229	1256
11	2.0	0.6	1414	1395	1426

NOTE: Incubation, isolation, and counting were carried out as described in Materials and Methods. Specific activity of glycine, 18.68 c.p.m./mμmole.
*Calculated assuming K_m = 6.55 mmolar, K_i = 2.12 mmolar, and A = 6148 c.p.m./aliquot, corresponding to a maximum concentration gradient of 53 mmolar.
†Calculated assuming K_m = 8.0 mmolar, K_i = 3.03 mmolar, and A = 8270 c.p.m./aliquot.

TABLE IV

A comparison of the theoretical and observed levels of radioactivity in aliquots of the alcohol-soluble fraction of ascites cells after attainment of steady-state levels of glycine-1-C^{14} in the presence of various amounts of this substrate and of 3-amino-3-carboxypropane sulphonamide

Vessel number	Glycine-1-C^{14} (mmolar)	3-Amino-3-carboxypropane sulphonamide (mmolar)	c.p.m./aliquot		
			Calculated* from equation [5]	Observed	Calculated† from simplified form of equation [5]
1	6.0	0	4880	4956, 4871	4960
2	3.0	0	3118	3066	3118
3	2.0	0	2299	2259, 2236	2270
4	1.6	0	1925	1813	1890
5	1.3	0	1618	1642	1580
6	1.0	0	1293	1306, 1222	1251
7	2.0	7.0	1125	1048	1047
8	2.0	6.0	1203	1081	1135
9	2.0	5.0	1298	1298	1239
10	2.0	4.0	1412	1342	1361
11	2.0	3.0	1554	1596	1512
12	2.0	2.0	1733	1776	1702
13	2.0	1.0	1972	1980	1942

NOTE: Incubation, isolation, and counting were carried out as described in Materials and Methods. Specific activity of glycine = 18.68 c.p.m./mμmole.
*Calculated assuming K_m = 6.24 mmolar, K_i = 4.02 mmolar, and A = 8520 c.p.m./aliquot, corresponding to a maximum concentration gradient of 73 mmolar.
†Calculated assuming K_m = 8.75 mmolar, K_i = 5.12 mmolar, and A = 12200 c.p.m./aliquot.

(a) Allyl Glycine

This compound was the most effective inhibitor of methionine transport of the analogues employed in the present experiments. The effects of allyl glycine

on the steady-state level of methionine inside ascites cells were investigated and the results obtained are presented in Fig. 3. Under conditions where transport of methionine is markedly inhibited, the simplified equation used by Cohen and Monod (3) and Tenenhouse and Quastel (4) is no longer applicable. This may explain why the latter authors observed deviations from linearity in their studies on the transport of the methionine isomer, S-ethylcysteine.

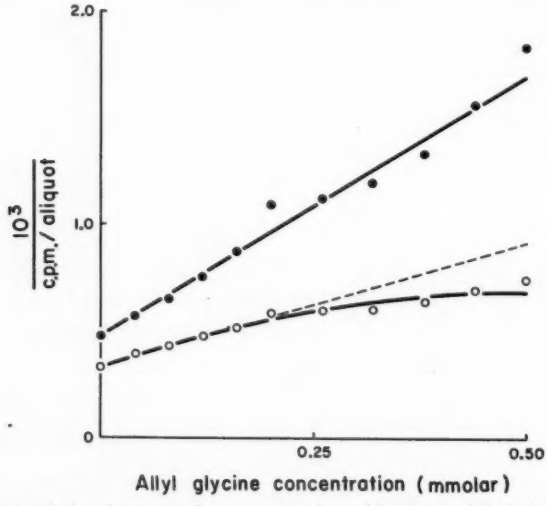

FIG. 3. The relation between the concentration of inhibitor (allyl glycine) and the reciprocal of the radioactivity per aliquot of the alcohol-soluble fraction of ascites cells after incubation with DL-methionine-S^{35} (specific activity, 64.47 c.p.m./mμmole DL-methionine): ○, no correction applied; ●, correction applied as indicated in the text. Incubation was carried out as described in Materials and Methods.

The effects of allyl glycine were analyzed by the standard kinetic methods discussed above, in which the concentration of DL-methionine-S^{35} and of allyl glycine were varied independently. It was anticipated that no values for A, K_m, and K_i would lead to figures in agreement with the observed results if the simplified form of equation [5] was used, but good agreement should be obtained if equation [5] itself was employed. The deviations should be most apparent at high concentrations of substrate or inhibitor, both being conditions which lead to a low concentration factor (a_c/a_f). The results obtained are presented in Table V. In calculating the values quoted in this table, the constants were so adjusted that the equations gave an accurate estimate of the uptakes in the absence of inhibitor and in the presence of the lowest concentration of allyl glycine. The use of equation [5] gives values for the extent of uptake of methionine in the presence of all concentrations of allyl glycine employed which are in good agreement with the observed values. The use of the simplified equation failed to do so and the error became as high as 20% when the highest

TABLE V

A comparison of the theoretical and observed levels of radioactivity in aliquots of the alcohol-soluble fraction of ascites cells after attainment of steady-state levels of DL-methionine-S^{35} in the presence of various amounts of this substrate and of allyl glycine

Vessel number	DL-Methionine-S^{35} (mmolar)	Allyl glycine (mmolar)	c.p.m./aliquot		
			Calculated* from equation [5]	Observed	Calculated† from simplified form of equation [5]
1	1.0	0	1647	1777	1615
2	1.0	0.6	1242	1219	1220
3	1.0	1.2	1033	993	981
4	1.0	2.0	866	866	778
5	1.4	0	2086	2083	2025
6	1.4	0.6	1631	1534	1601
7	1.4	1.2	1379	1395	1302
8	1.4	2.0	1174	1157	1065
9	2.0	0	2621	2658	2660
10	2.0	0.6	2136	2145	2098
11	2.0	1.2	1850	1795	1731
12	2.0	2.0	1604	1752	1405
13	4.0	0	3924	3925	3930
14	4.0	0.6	3469	3434	3275
15	4.0	1.2	3151	3211	2810
16	4.0	2.0	2849	2969	2370

NOTE: Incubation, isolation, and counting were carried out as described in Materials and Methods. Specific activity of DL-methionine, 55.53 c.p.m./mμmole.

*Calculated assuming K_m = 1.85 mmolar, K_I = 0.86 mmolar, and A = 3710 c.p.m./aliquot, corresponding to a maximum concentration gradient of 10.7 mmolar.

†Calculated assuming K_m = 3.64 mmolar, K_I = 1.45 mmolar, and A = 7500 c.p.m./aliquot.

concentrations of allyl glycine and DL-methionine were used. The applicability of equation [5] indicates that the transport of DL-methionine into ascites cells is competitively blocked by allyl glycine and that its diffusion out proceeds at a rate which is proportional to the concentration gradient of methionine between the cells and the medium.

(b) 1-Aminocyclopentane Carboxylic Acid, 3-Amino-3-carboxypropane Sulphonamide, and Furyl Glycine

The results obtained with these three analogues are presented in Tables VI, VII, and VIII. The observed values for the Michaelis-type constant for DL-methionine are in good agreement with that reported in Table V.

(iii) DL-*Leucine-1-*C^{14}

Incubation of ascites cells in a medium containing 2 mmolar glycine or 2 mmolar DL-methionine led to a concentration of glycine by the cells to the extent of approximately sevenfold and of DL-methionine to the extent of three- to four-fold.

Preliminary experiments confirmed that DL-leucine is poorly concentrated by ascites cells (4, 12) and that the concentration factor (a_c/a_t) is dependent on the concentration of leucine in the incubation medium (Table IX). The concentration gradient is too small to permit accurate kinetic analysis of the leucine transport system but sufficient to investigate qualitatively the ability

TABLE VI

A comparison of the theoretical and observed levels of radioactivity in aliquots of the alcohol-soluble fraction of ascites cells after attainment of steady-state levels of DL-methionine-S^{35} in the presence of various amounts of this substrate and of 1-aminocyclopentane carboxylic acid

Vessel number	DL-Methionine-S^{35} (mmolar)	1-Aminocyclopentane carboxylic acid (mmolar)	c.p.m./aliquot	
			Calculated* from equation [5]	Observed
1	1.0	0	1887	1928
2	1.0	1.6	1516	1553
3	1.0	3.2	1284	1357
4	1.0	5.0	1110	1109
5	1.4	0	2359	2337
6	1.4	1.6	1958	Lost
7	1.4	3.2	1685	1678
8	1.4	5.0	1483	1417
9	2.0	0	2948	2914
10	2.0	1.6	2520	2382
11	2.0	3.2	2217	2229
12	2.0	5.0	1980	1944
13	4.0	0	4308	4308
14	4.0	1.6	3925	4006
15	4.0	3.2	3620	3758
16	4.0	5.0	3347	3284

NOTE: Incubation, isolation, and counting were carried out as described in Materials and Methods. Specific activity of DL-methionine-S^{35}, 54.01 c.p.m./mμmole.

*Calculated assuming K_m = 1.76 mmolar, K_I = 3.22 mmolar, and A = 4260 c.p.m./aliquot, corresponding to a maximum concentration gradient of 12.6 mmolar.

TABLE VII

A comparison of the theoretical and observed levels of radioactivity in aliquots of the alcohol-soluble fraction of ascites cells after attainment of steady-state levels of DL-methionine-S^{35} in the presence of various amounts of this substrate and of 3-amino-3-carboxypropane sulphonamide

Vessel number	DL-Methionine-S^{35} (mmolar)	3-Amino-3-carboxypropane sulphonamide (mmolar)	c.p.m./aliquot	
			Calculated* from equation [5]	Observed
1	1.0	0	2879	2608
2	1.0	3.0	2228	2349
3	1.0	7.0	1734	1743
4	1.0	12.0	1408	1339
5	1.3	0	3349	3364
6	1.3	3.0	2904	2851
7	1.3	7.0	2346	2207
8	1.3	12.0	1934	1801
9	2.0	0	4178	4179
10	2.0	3.0	3506	3690
11	2.0	7.0	2928	2910
12	2.0	12.0	2473	2526
13	4.0	0	5667	5907
14	4.0	3.0	5127	5036
15	4.0	7.0	4577	4410
16	4.0	12.0	4087	4001

NOTE: Incubation, isolation, and counting were carried out as described in Materials and Methods. Specific activity of DL-methionine-S^{35}, 55.87 c.p.m./mμmole.

*Calculated assuming K_m = 1.19 mmolar, K_I = 4.65 mmolar, and A = 5550 c.p.m./aliquot, corresponding to a maximum concentration gradient of 15.8 mmolar.

TABLE VIII

A comparison of the theoretical and observed levels of radioactivity in aliquots of the alcohol-soluble fraction of ascites cells after attainment of steady-state levels of DL-methionine-S^{35} in the presence of various amounts of this substrate and of furyl glycine

Vessel number	DL-Methionine-S^{35} (mmolar)	Furyl glycine (mmolar)	cp..m./aliquot	
			Calculated* from equation [5]	Observed
1	1.0	0	2635	2685
2	1.0	1.0	1348	1389
3	1.0	2.0	1073	1036
4	1.0	4.0	829	768
5	1.4	0	3326	3230
6	1.4	1.0	1925	1744
7	1.4	2.0	1467	1438
8	1.4	4.0	1116	1127
9	2.0	0	4155	4087
10	2.0	1.0	2585	2551
11	2.0	2.0	2029	2063
12	2.0	4.0	1570	1560
13	4.0	0	6100	6362
14	4.0	1.0	4450	4694
15	4.0	2.0	3702	3854
16	4.0	4.0	3005	3311

NOTE: Incubation, isolation, and counting were carried out as described in Materials and Methods. Specific activity of DL-methionine-S^{35}, 69.21 c.p.m./mμmole.
*Calculated assuming K_m = 1.95 mmolar, K_i = 0.54 mmolar, and A = 6500 c.p.m./aliquot, corresponding to a maximum concentration gradient of 15.0 mmolar.

TABLE IX

The concentration of DL-leucine-1-C^{14} by Ehrlich ascites cells

Concentration of DL-leucine-1-C^{14} in the medium (mmolar)	c.p.m./aliquot of alcohol-soluble fraction	a_e (mmolar)	a_e/a_f
6.0	1218	7.00	1.16
3.0	704	4.05	1.35
2.0	495	2.84	1.42
1.6	411	2.37	1.48
1.3	376	2.16	1.66
1.0	290	1.67	1.67

NOTE: Incubation, isolation, and counting were carried out as described in Materials and Methods. The specific activity of DL-leucine was 27.85 c.p.m./mμmole. The figures quoted are mean values obtained from duplicate flasks in the same experiment.

of other amino acids or their analogues to inhibit the transport of leucine. The effects of 1-aminocyclopentane carboxylic acid on the transport of DL-leucine were investigated since this compound is an effective inhibitor of transport of glycine and DL-methionine. The results obtained are presented in Table X. The analogue increases, rather than decreases, the amount of DL-leucine accumulated by the ascites cells, a concentration of 0.4 mmolar being sufficient to bring about an increase of 42%. At higher concentrations (0.8–3.0 mmolar) there is a constant stimulation of DL-leucine uptake of 62% (range 52–71%). In another experiment, it was shown that, at concentrations greater than 4 mmolar, 1-aminocyclopentane carboxylic acid causes decreasing stimulations

of leucine uptake and in the presence of 10 mmolar analogue the stimulation is only 27%. In the first experiment the incorporation of DL-leucine into the alcohol-insoluble fraction was also measured and the results obtained are also presented in Table X.

TABLE X

The effects of 1-aminocyclopentane carboxylic acid on the uptake of DL-leucine-1-C^{14} by Ehrlich ascites carcinoma cells

Expt.	Concentration of 1-aminocyclopentane carboxylic acid added (mmolar)	Uptake of DL-leucine-1-C^{14} (μmoles/ml packed cells) (alcohol soluble)	Incorporation of DL-leucine-1-C^{14} (μmoles/ml packed cells) (alcohol insoluble)
1	0	2.49, 2.34 (100)	0.44, 0.38 (100)
	0.4	3.44 (142)	0.43 (102)
	0.8	3.95 (163)	0.39 (93)
	1.2	3.99, 3.81 (162)	0.40, 0.32 (86)
	1.6	3.67 (152)	0.37 (88)
	2.0	3.75 (155)	0.37 (88)
	2.5	4.14 (171)	0.37 (88)
	3.0	4.10 (169)	0.34 (81)
2	0	2.34, 2.36 (100)	—
	2.0	3.79, 3.79 (161)	—
	4.0	3.78, 3.66 (158)	—
	6.0	3.70, 3.39 (151)	—
	8.0	3.29, 3.31 (140)	—
	10.0	2.90, 3.10 (127)	—

NOTE: The concentration of DL-leucine used was 2 mmolar; specific activity in experiment 1, 22.38 c.p.m./mμmole; and in experiment 2, 27.85 c.p.m./mμmole. Incubation, isolation and counting were carried out as described in Materials and Methods. The figures in parentheses refer, in each case, to percentages of the mean value of the controls.

To determine whether the effect on DL-leucine uptake involves an effect on the rate of uptake, a time-course study was undertaken. The results obtained are presented in Fig. 4. They show that 1-aminocyclopentane carboxylic acid has little effect on the velocity of uptake of leucine in the first 5 minutes of incubation. However, in the presence of this analogue, the uptake of DL-leucine

TABLE XI

The effects of 1-aminocyclopentane carboxylic acid on the oxidation of DL-leucine-1-C^{14} by Ehrlich ascites carcinoma cells

Concentration of 1-aminocyclopentane carboxylic acid added (mmolar)	Oxygen taken up in 50 minutes (μl)	Leucine oxidized* in 60 minutes (mμmoles)	Oxygen equivalent to leucine oxidized (μl)†
0	149	166	27.8
0.4	145	167	28.0
1.2	134	177	29.7
2.0	137	185	31.0

NOTE: Ehrlich ascites cells were incubated for 1 hour with 2 mmolar DL-leucine-1-C^{14} (specific activity, 16.5 c.p.m./mμmole) and the oxygen uptakes measured over the period 5–55 minutes. At the end of the incubation period, the reaction was terminated by addition of trichloracetic acid and the radioactive carbon dioxide collected and counted as described in Materials and Methods.

*Calculated from the formula mμmoles leucine oxidized = (c.p.m. of $BaCO_3$)/(c.p.m./mμmole leucine).

†Calculated assuming that the radioactive carbon dioxide collected is a measure of complete oxidation of DL-leucine.

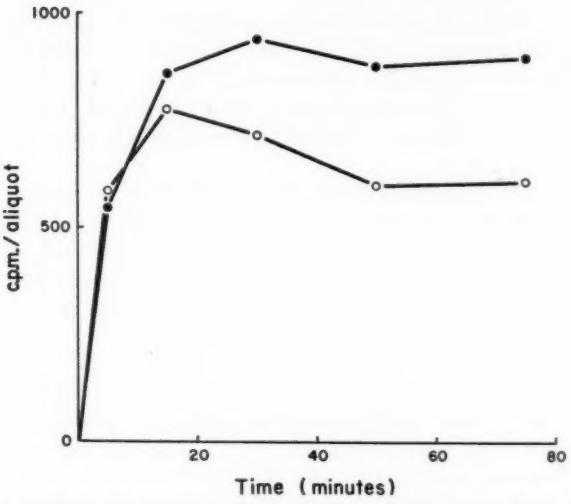

FIG. 4. The effects of 2 mmolar 1-aminocyclopentane carboxylic acid on the uptake of leucine by Ehrlich ascites cells from a medium containing 2 mmolar DL-leucine-1-C^{14} (specific activity, 22.4 c.p.m./mμmole): ○, no inhibitor present; ●, 2 mmolar 1-aminocyclopentane carboxylic acid present.
Incubation was carried out as described in Materials and Methods.

continues to increase after that time, reaches a maximum after 15–20 minutes, and remains at that level. In the absence of the analogue, the uptake increases to a maximum after 15 minutes' incubation and subsequently decreases. This type of phenomenon has not previously been observed in time-course studies on the uptake of glycine or DL-methionine (5, 13) and suggests that a metabolism of DL-leucine-1-C^{14} may be involved. The rate of oxidation of this amino acid was studied and the results obtained are presented in Table XI. There is a

TABLE XII

The effects of other amino acids and amino acid analogues on the uptake of DL-leucine-1-C^{14} by Ehrlich ascites carcinoma cells

Amino acid added	c.p.m./aliquot
Nil	309 (100)
Valine	316 (102)
Norvaline	396 (128)
Isoleucine	279 (90)
Norleucine	522 (169)
1-Aminocyclopentane carboxylic acid	497 (161)
N-Methyl-2-phenyl glycine	276 (89)
Allyl glycine	426 (138)
3-Amino-3-carboxypropane sulphonamide	437 (141)
Thienyl glycine	246 (80)

NOTE: Incubation was carried out as described in Table X. Specific activity of the leucine was 21.0 c.p.m./mμmole. The figures in parentheses refer to percentages of the control value obtained in the presence of 2 mmolar DL-leucine-1-C^{14} only.

rapid oxidation of DL-leucine-1-C^{14}, as measured by the production of radioactive CO_2, in agreement with the results obtained by Emmelot and van Vals (14) with an ascitic form of a mammary carcinoma. However, instead of an inhibition by 1-aminocyclopentane carboxylic acid, a stimulation of radioactive CO_2 production was obtained without a corresponding effect on respiratory activity.

The effects of several other natural and unnatural amino acids on the uptake of DL-leucine-1-C^{14} were investigated and the results obtained are presented in Table XII. At a concentration of 2 mmolar, several of the other amino acids stimulate the uptake as presently measured and small but significant inhibitory effects are obtained with DL-isoleucine, N-methyl-2-phenyl glycine, and thienyl glycine.

Discussion

In the present analysis of the kinetics of amino acid transport into Ehrlich ascites carcinoma cells, it has been assumed that only two factors influence the steady-state levels of amino acids inside the cells. One factor is a component, or phase, with which the amino acids must be associated before entering the amino acid pool of the cells, which is present in limited quantities and whose continued presence is dependent on a supply of energy. It seems most likely that this factor is, in fact, a carrier for the amino acids of the type postulated by Heinz and Walsh (15). Other mechanisms exist, which may be involved in the entry of amino acids into the ascites carcinoma cells, such as exchange diffusion (13, 15), but such mechanisms either do not alter the steady-state levels of the individual amino acids or are not operating under the present experimental conditions.

The other factor considered is that involved in the loss of amino acids from the ascites carcinoma cells. Exchange diffusion is again a potential factor but apparently does not alter the steady-state levels in the experiments discussed. The most important factor, possibly the only one, involved in the loss of amino acids appears to be diffusion. Such diffusion obeys Fick's law and is proportional not to the concentration of the particular amino acid in the cell but to the concentration gradient between the cells and the medium. As a result, diffusion may occur *into* the cells at the onset of incubation but once the amino acid has been concentrated by the cells, diffusion *out* of the cells will begin.

If these are the only two factors involved in the present experiments, then the inhibitory action of other amino acids must be due to competition by them for the limited amount of the carrier component whose function is defined above since they cannot influence simple diffusion. Competitions of this type should obey the same mathematical law as that which characterizes competitive inhibition of enzyme-catalyzed reactions. This has been shown to be true and good agreement obtained between observed and theoretical values calculated from this formula. A summary of the values of the constants, so derived, is shown in Table XIII. These values support the conclusion drawn previously

TABLE XIII

The constants characterizing the competitive inhibition of the transport of glycine and DL-methionine by other amino acids

Substrate	K_m (mmolar)	K_i (mmolar)				Maximum concentration gradient (mmolar)
		1-Aminocyclopentane carboxylic acid	Allyl glycine	3-Amino-3-carboxy-pentane sulphonamide	Furyl glycine	
Glycine	6.40	1.47	—	—	—	49
	6.55	—	2.12	—	—	53
	6.24	—	—	4.02	—	73
DL-Methionine	1.76	3.22	—	—	—	12.6
	1.85	—	0.86	—	—	10.7
	1.19	—	—	4.65	—	15.8
	1.95	—	—	—	0.54	15.0

(13) that glycine and methionine are transported into the cells by quite separate systems. The propane sulphonamide derivative has about the same affinity for both transport systems, but the affinity of the methionine transport system for allyl glycine is more than twice that of the glycine transport system for the same compound. The opposite is true when the affinities of the two systems for 1-aminocyclopentane carboxylic acid are compared. Further, thienyl glycine has little effect on glycine transport but is the most effective inhibitor of methionine transport among the compounds studied.

The various determinations of the K_m values, characteristic of glycine or of DL-methionine transport, are quite consistent among the various typical experiments quoted. The maximum concentration gradients (49, 53, and 73 mmolar) for glycine are in reasonable agreement with the value of 45 ± 14 mmolar found by Heinz and Mariani (10) but the average K_m value (6.4 mmolar) is considerably greater than the value of 3.7 mmolar found by Heinz (2) and Tenenhouse and Quastel (4). Later results obtained by the latter authors, using cells from the same groups of animals as those used in the present experiments, gave results similar to those reported above.

Transport of DL-leucine is so slow (or its diffusion is so rapid) that little accumulation of this amino acid occurs in ascites carcinoma cells. An alternative hypothesis is that DL-leucine-1-C^{14} is oxidized so rapidly that it cannot accumulate and previous determinations (14) have shown that $C^{14}O_2$ is produced quite rapidly from this substrate. The present experiments show (Table XI) that DL-leucine is oxidized at the rate of 1.33 μmoles/hr/ml packed cells, assuming that the production of radioactive CO_2 is an index of the complete oxidation of DL-leucine. This is of the same order as the initial velocity of uptake of DL-leucine which is 2.43 μmoles/hr/ml packed cells, calculated from the values for the first 5 minutes presented in Fig. 4. The amount of DL-leucine accumulating within the Ehrlich ascites cells in the presence of other amino acids is, in many cases, increased (Tables X and XII). This could be explained by an inhibition of the oxidation of DL-leucine but, as shown in Table XI, the stimulation of amino acid uptake is accompanied by a somewhat smaller stimulation of DL-leucine oxidation on addition of 1-aminocyclopentane carboxylic acid. There may, therefore, be a true stimulation of amino acid uptake which, in turn, leads to a more rapid rate of oxidation of the leucine.

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11-KETOTESTOSTERONE: AN ANDROGEN FOR SOCKEYE SALMON[1]

D. R. IDLER,[2] I. I. BITNERS, AND P. J. SCHMIDT

Abstract

11-Ketotesterone, a hormone in the blood of sockeye salmon, has been shown to have androgenic activity for this species. It influences skin thickness and coloration, flesh pigmentation, and spermatogenesis in the male. The effect of 11-ketotestosterone is not so pronounced in the female but it influences both skin thickness and coloration. Estradiol increased the mass of the female gonads.

Introduction

An androgen is a substance that promotes the development of secondary sex characteristics. A previous study has demonstrated that 11-ketotestosterone is a potent androgenic hormone as determined by the chick-comb bio-assay (1). This steroid has been isolated, in this laboratory, from the plasma of sexually mature male salmon (2). A substance with the chromatographic mobility of 11-ketotestosterone occurs in high concentration in the plasma of sexually mature female sockeye salmon and is the major adrenocorticosteroid in the plasma of spawned *Salmo salar* males (P.J. Schmidt and D. R. Idler. Submitted for publication).

It was the purpose of the present investigation to determine if 11-ketotestosterone was an androgen for salmon.

The most pronounced secondary sex characteristics of the male sockeye are an intense red coloration of the skin, an elongation of the snout, and the development of a hump on the back. The female undergoes these changes to a much lesser degree than does the male. Less obvious manifestations of increasing sexual maturity are a thickening of the skin and a decrease in the amount of carotenoid pigments (3) in the flesh.

Methods

Several groups of sexually immature sockeye salmon were taken prior to their entrance into Great Central Lake on Vancouver Island during the latter part of June and early July, 1960. These fish were transferred to the outdoor holding ponds of the B.C. Fish and Game Department at Courtenay, B.C., where procedures were developed for holding the fish in good condition in spite of the high water temperatures (4).

The fish used for this experiment were transported to Courtenay on July 10, 1960, and given salt baths, to prevent fungus infection, every 2 days until the experiment was begun on July 20. On July 20, 43 fish were divided into two

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[2]Present address: Fisheries Research Board of Canada, Technological Station, Halifax, N.S.

groups and each group was held in an identical tank. The fish from one group (predominantly male) were anaesthetized with MS-222,[3] 44.5 p.p.m. at 9–11° C, and injected intramuscularly in the vicinity of the dorsal fin with 5 mg of 11-ketotestosterone in 0.2 ml of peanut oil. The oil was injected through a No. 18 3-in. needle which had previously been dipped in penicillin G (ca. 1.2×106 units/ml). The control groups were given injections of peanut oil. The injections were made at weekly intervals.

A single fish from the control group was implanted, 5 days after the experiment began, with a pellet containing 37 mg of 11-ketotestosterone and no added carrier. A longitudinal subcutaneous incision of the same width as the pellet was made for a length of about $1\frac{1}{2}$ inches in the side of the fish and instruments were dipped in penicillin to minimize risk of infection. On August 2, 13 days after the beginning of the injection in the initial experiment, four additional fish from the control group were started on injections of 11-ketotestosterone. These four fish and the fish given the pellet were marked with yellow tags. On the same date five of the control group (four females and one male) were injected with estradiol and these injections were continued at weekly intervals (5 mg/0.2 ml of peanut oil). These fish were marked with red tags.

The tanks were checked at frequent intervals and dead fish were removed immediately. Post-mortem storage experiments carried out in this laboratory have demonstrated that flesh color is not significantly affected by the short post-mortem storage encountered in these experiments.

Flesh color was measured on freshly cut steaks and this and the other determinations were made on a steak taken from the same location in each fish. Fat and moisture determinations were performed on the flesh after canning (5).

Results and Discussion

At the end of 2 weeks there was a very significant visual difference in the appearance between the group injected intramuscularly with 11-ketotestosterone and the control group. A photographic record was obtained after the experiment had been in progress for 19 days. The fish in the control group still had the silvery appearance of "green" fish, while all the male fish which had been injected were colored an intense red. On August 17, when the experiment had been in progress for 28 days, further pictures were obtained. The fish in the control group still had their silvery appearance, while the males which had been injected were already beginning to show a darkening of their skin which a week before had been bright red. The injected fish were also beginning to show a pronounced elongation of the snout. At this time it was difficult to distinguish the males from the females in the control group but post-mortem examination (Table I) showed that there were males in the control group (blue tags and no tag).

By the time the experiment had been in progress for 28 days it became apparent that there was no observable difference in the appearance of the control

[3]Tricaine methanesulphonate available from Sandoz Pharmaceuticals, Montreal, Que.

TABLE I

Effect of 11-ketotestosterone and estradiol on salmon

No. or tag	Sex	% moisture	% fat	Gonads (% live wt. of fish)	Color reading (*a*)	Skin thickness (mm)	Death	Egg size (mm)
Green, group I*								
8	M	78.8	2.63	0.37	0.6	.762	Sept. 7(K)	
6	M	79.2	2.59	1.08	4.3	.762	Sept. 7(K)	
7	M	78.8	2.63	1.00	4.2	.838	Sept. 7(K)	
1	F	76.4	4.35	5.90	2.9	.711	Sept. 7(K)	3.5–4.0
21	M	81.0	2.92	0.68	2.4	.965	Sept. 19(D)	
Green, group II†								
23	M	76.0	3.07	0.88	10.8	.686	Aug. 19(D)	
4	F	77.6	3.69	5.03	7.6	.737	Sept. 7(K)	
12	M	76.2	3.29	0.41	5.9	.940	Sept. 7(K)	
11	M	76.2	3.29	0.58	8.0	.864	Sept. 7(K)	
9	F	77.6	3.69	7.80	3.3	.686	Sept. 7(K)	4.0–4.5
3	M	78.4	2.33	0.60	5.5	.864	Sept. 7(K)	
24	F	77.0	2.78	5.86	26.1	.660	Aug. 25(D)	4.5
25	F	73.6	5.55	4.48	27.4	.711	Aug. 25(D)	4.0
28	M	74.0	4.34	0.18	18.2	.711	Aug. 23(D)	
37	F	75.6	5.30	10.23	6.2	.864	Sept. 6(D)	4.5–5.0
38	M	—	—	0.51	1.4	.737	Sept. 29(D)	
Red tag*								
22	F	74.0	4.87	12.21	32.4	.508	Aug. 19(D)	
26	F	77.0	2.60	13.93	32.0	.533	Aug. 22(D)	5.0
34	M	72.8	2.99	0.28	27.2	.559	Aug. 24(K)	
2	F	76.6	2.70	20.70	25.7	.533	Sept. 7(K)	6.0
20	F	81.0	2.06	28.10	9.8	.610	Sept. 17(D)	5.5–6.0
Yellow tag*								
35	M	75.8	2.21	0.92	11.7	.914	Aug. 24(D)	
36	M	74.6	2.98	1.60	23.6	.635	Aug. 24(D)	
5‡	F	77.3	3.31	6.70	5.0	.762	Sept. 7(K)	4.0–4.5
10	F	74.2	5.86	5.66	11.8	.787	Sept. 7(K)	4.0
19	F	79.0	3.05	11.70	4.8	.864	Sept. 11(D)	5.5–6.0
Blue tag*								
15	M	73.0	4.91	2.80	35.8	.610	Sept. 7(K)	
27	F	74.0	3.33	10.28	30.4	.610	Aug. 22(D)	5.0–5.5
34	F	73.6	3.16	8.12	34.2	.685	Aug. 23(K)	4.0–4.5
No tag*								
29	F	74.2	2.22	11.26	29.0	.508	Aug. 23(K)	4.5–5.0
32	F	73.6	5.79	8.90	27.6	.559	Aug. 23(K)	4.0
13	F	71.6	6.25	6.00	35.6	.635	Sept. 7(K)	3.5
14	M	73.0	4.91	1.70	37.0	.635	Sept. 7(K)	
16	F	71.6	6.25	9.00	36.1	.432	Sept. 7(K)	4.0
17	F	71.6	4.90	6.30	35.8	.584	Sept. 7(K)	3.8
18	F	72.6	4.28	8.50	39.9	.584	Sept. 7(K)	4.0–4.5
30	M	73.8	2.90	3.00	32.3	.457	Aug. 23(K)	
31	M	72.0	2.52	2.07	27.0	.584	Aug. 23(K)	
39	F	—	—	12.00	28.3	.508	Oct. 25(D)	4.5–5.0
40	F	—	—	19.00	—	—	Dec. 15(D)	4.5
41	F	—	—	14.00	—	—	Dec. 15(D)	4.0–4.5
42	F	—	—	12.60	—	—	Jan. 2(D)	4.0–4.5
43	M	—	—	2.03	—	—	Jan. 13(D)	

NOTE: K, killed; D, died.

*Green tag group I fish given 11-ketotestosterone injections weekly from July 20 until death. Red tag fish injected with estradiol from August 2 until death. Blue tag fish injected with peanut oil only from July 20 until death. Yellow tag fish, except for No. 5, were given 11-ketotestosterone injections weekly beginning August 2. No tag fish injected with peanut oil only from July 20 until August 17.

†11-Ketotestosterone injections stopped August 17.

‡A 37-mg pellet of 11-ketotestosterone implanted in this fish.

fish which had been receiving peanut oil and those which were not being injected. These fish were continued on peanut oil injection until they died or were killed and these were given a blue tag. For the remainder the peanut oil injections were discontinued.

The fish were moved from Courtenay, B.C., to the Vancouver Public Aquarium on August 17 (28 days after the start of 11-ketotestosterone injections).

On August 17 (15 days after the beginning of the estrogen injections) the female estrogen-injected fish had a much darker coloration in the belly region than did the controls, and they had also developed a yellow coloration particularly in the peduncle area. The male fish which were given their first injection on August 2 and the one fish which received a pellet (yellow tag) showed well-developed secondary sex characteristics and the lone female (yellow tag) had a moderate red coloration of the skin and a slight yellowing around the anus. At this time two of the fish, later shown by autopsy to be a male and female, in the control group showed a red coloration of the skin. However, it was not so intense as the coloration of the 11-ketotestosterone injected fish several weeks earlier and it was not present to any significant extent on the underside of the fish.

After August 17, injections of 11-ketotestosterone were discontinued except to a small group composed of four males and one female (green tag, group I, Table I).

The observations made in the field and a study of the color photographs which were taken clearly showed that 11-ketotestosterone was an androgenic substance for salmon. However, it was considered desirable to attempt to obtain some quantitative expression of this fact. It was felt that flesh color, skin thickness, and gonad development might be suitable criteria. However, it was necessary to sacrifice the fish in order to obtain this information. The problem was further complicated by the fact that some of the fish died from time to time and it was difficult to sacrifice sufficient numbers at any one time to be sure that the results would be significant. Fortunately, the differences between the injected and the control fish of both sexes were of a sufficiently large order of magnitude and sufficiently consistent that certain conclusions can be drawn. The data obtained for fish sacrificed on September 6 and 7 are shown in Table II. In a previous study carried out in this laboratory it has been shown that the color of the flesh expressed as the "*a*" value on the Gardner Automatic Color Difference Meter[4] is a good quantitative measure of the red pigmentation of the flesh (6). The flesh of prime quality immature sockeye generally gives an "*a*" value in excess of 30. Both male and female fish in the control group had "*a*" values over 30 and by contrast all 11-ketotestosterone injected fish had "*a*" values under 10. Flesh with an "*a*" value of less than 10 has at the most only traces of red pigment. The three male fish which received their last injection of 11-ketotestosterone on August 17 (3 weeks before death)

[4]Manufactured by Gardner Laboratory Inc., Bethesda 14, Maryland, U.S.A.

TABLE II

Effect of 11-ketotestosterone on sockeye sacrificed September 7

Rx	Sex	Fish	Flesh color "*a*"*	Skin (mm)	Gonads† (%)	Sperm‡
Steroid	M	6§	0.6–8.0	0.76–0.94	0.37–1.08	+
None	M	2	36, 37	0.61–0.64	1.7 –2.8	–
Steroid	F	4‖	2.9–7.6	0.69–0.86	5–10	
None	F	4	36, 40	0.43–0.63	6–9	

*Value by Hunter Color Difference Meter.
†Gonads expressed as percentage of the live weight of the fish.
‡Microscopic examination of spermatic fluid.
§Last injection of three fish on August 17.
‖One fish sacrificed September 6. Last injection of fish on August 17.

had an average flesh "*a*" value of 3.0 as compared with 6.5 for the three males which received 11-ketotestosterone until 1 week before death. All male fish which continued to receive injections had lower flesh "*a*" values than those for which the injections were stopped (Table I). The male fish sacrificed on August 19–25 also showed much lower flesh "*a*" values for the 11-ketotestosterone-injected than for the control. While there appeared to be a slight difference at this time between the color of the flesh of injected and control females, it was not nearly so pronounced as it was with the fish sacrificed on September 7.

Both male and female 11-ketotestosterone-injected fish sacrificed on September 7 had thicker skins than those of the controls. The same relationship also held for males sacrificed on October 19–25 but, as was the case with color, the effect on females was not so great for those fish sacrificed on the earlier dates.

The ratio of the weight of the gonads to the live weight of the fish has been shown to be a sensitive indicator of sexual maturity in investigations carried out on the Fraser River sockeye (5). This expression of maturity was particularly useful in determining the sexual maturity of females. The gonads of the 11-ketotestosterone-injected males were smaller than were the controls for fish sacrificed on September 7 (Table II). This was also true for males sacrificed August 19–25 (Table III). There was no discernible difference in the size of the gonads of injected or control females sacrificed on September 7. However, only one fish received 11-ketotestosterone injections after August 17. There was a difference in the size of the gonads of females sacrificed on August 19–25,

TABLE III

Effect of 11-ketotestosterone on sockeye sacrificed August 19–25*

Rx	Sex	Fish	Flesh color "*a*"	Skin (mm)	Gonads (%)
Steroid	M	2	10.8, 18.2	.69, .71	.18, .88
None	M	2	27.0, 32.3	.46, .58	2.07, 3.0
Steroid	F	2	26.1, 27.4	.66, .71	4.5, 5.9
None	F	4	27.6, 34.2	.51, .69	8.1, 11.3

*Male and female control fish sacrificed August 23. One male injected fish died on August 19 and the other August 23. Three female controls were killed August 23 and one died August 22.

with the gonads of 11-ketotestosterone-injected females being smaller than those of the control fish. The data for females are not as conclusive as those for males.

The testes of males sacrificed on September 7 contained the milky fluid characteristic of sexually mature fish and microscopic examination showed that sperm were present. There was no significant amount of spermatic fluid in the testes of control fish sacrificed up until September 7 but it was present in the steroid-injected males sacrificed on August 19–25.

There also appeared to be some correlation between the color of the liver of the fish at death and the treatment which the fish had received. With the exception of a single male, all fish which were injected with 11-ketotestosterone varied in color from green brown to dark green. Livers of the control fish were all brown with the exception of two females which were green and brown. The estrogen-injected females also had brown livers.

The data for the fish receiving the pellet of 11-ketotestosterone and injections which did not begin until August 2 (yellow tag, Table I) confirm that females injected with 11-ketotestosterone and sacrificed September 7 and 11 had very little color in their flesh. It therefore seems reasonable to conclude that the loss of color from the female flesh occurred to the greatest extent between August 17 and September 7.

Three females which died on December 25, 1960, or January 2, 1961, had gonads which averaged 15.2% of the live weight of the fish and the eggs were 4.0–4.5 mm in diameter. Another female which died on October 25 had gonads which were 12.0% of the live weight of the fish and the eggs were 4.5–5.0 mm. The gonads of six females killed on August 23 or September 7 averaged 8.3% of the live weight of the fish for the controls and the eggs were approximately 4 mm in diameter. The effect of estradiol injections on the size of the gonads was already apparent by August 19–22 when the gonads of two hormone-treated fish averaged 13.1% of the live weight. However, the estrogen-injected females sacrificed on September 7 and 17 had gonads which weighed 21 and 28%, respectively, of the live weight of the fish. These latter fish had eggs which were 5.5–6.0 mm in diameter. The more advanced maturity of the 11-ketotestosterone-injected fish was, on the average, also reflected in their high body water and low body fat content relative to the control group (Table I) (6).

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THE EFFECT OF SEROTONIN (5-HYDROXYTRYPTAMINE) ON THE OXIDATION OF C^{14} GLUCOSE TO $C^{14}O_2$ BY MOUSE LIVER, SPLEEN, AND KIDNEY SLICES[1]

P. V. Vittorio, D. L. Small, and M. J. Allen

Abstract

Nonirradiated and X-irradiated mice were killed 4 or 24 hours after the injection of saline or a protective dose of serotonin and the livers, spleens, and kidneys were removed, sliced, and incubated for 3 hours in a buffered medium containing uniformly labelled C^{14} glucose. The administration of serotonin to mice 4 hours before they were sacrificed resulted in an increased ability of the tissues to oxidize glucose to CO_2. Liver showed the greatest increase and spleen the smallest. X-Irradiation of the mice also increased the ability of the tissues to oxidize glucose to CO_2. However, the administration of serotonin to X-irradiated mice did not further increase the ability of the tissues to oxidize glucose except in liver, in which a slight effect was observed.

Introduction

It has been shown by several groups of investigators that serotonin (5-hydroxytryptamine), a compound that occurs in living organisms and is distributed in many parts of the body, provides good protection against radiation death in mice and rats (1, 2, 3, 4). Although much work has been done to show that serotonin is a radioprotective agent, very little is known regarding its mode of action in giving protection against radiation. We have investigated the effect of serotonin on thyroid activity in nonirradiated and X-irradiated rats and have shown that serotonin increased I^{131} uptake by the thyroids and that this was more pronounced in nonirradiated animals (5). The present study was carried out to determine the effect of serotonin injected in vivo on the in vitro respiration of mouse liver, spleen, and kidney slices.

Materials and Methods

Male mice weighing 25 ± 2.0 g were used. The serotonin solution was freshly prepared in physiological saline (0.9%) and neutralized with NaOH immediately prior to use. The required dose of serotonin (10 μmoles in 0.2 ml) was administered intraperitoneally. All mice were fasted during the experimental period, but they were permitted water ad libitum.

Irradiation of Animals

Fifteen minutes after the injection of serotonin or saline the mice were irradiated with a total body dose of 625 r. X-Irradiation was carried out using a Mueller X-ray machine operating at 300 kvp, 10 ma, delivering an air dose of 29.7 r per minute. The filtration employed was Al 0.133 g/cm^2 and Cu 0.888 g/cm^2. During irradiation, the mice were enclosed in a circular lucite cage

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which was rotated at 4 revolutions per minute. The nonirradiated control mice were also rotated in a similar lucite cage at the same rate.

Incubation Procedures

The mice were killed by a blow on the head; the livers, spleens, and kidneys were immediately removed and chilled. Slices 0.5 mm thick were prepared with a McIlwain mechanical tissue slicer. The tissues from 12 mice of the same group were sliced and pooled and 500 mg of liver, spleen, or kidney was placed in the outer compartment of a 50-ml incubation flask which contained uniformly labelled C^{14} glucose dissolved in 5.0 ml of buffered medium (6). The uniformly labelled glucose was prepared by the authors according to the method of Vittorio (7). For each group, incubations were carried out simultaneously in triplicate and each result represents the average value obtained. The flasks were gassed with a mixture of 95% O_2 and 5% CO_2. They were then capped with a self-sealing rubber stopper and incubated in a Dubnoff metabolic shaker (37.5° C) for 3 hours. At the end of the incubation period approximately 0.5 ml of 10% KOH was injected into the center well of the flask by means of a long needle attached to a hypodermic syringe. In a similar manner 1 *N* HCl was added to the incubation medium (pH 3) to inactivate the tissue.

At least 30 minutes was allowed for CO_2 absorption. The contents of the center well were then transferred to a round-bottom flask and the CO_2 was released *in vacuo* by the addition of acid and trapped in dilute (0.05 *N*) NaOH. The CO_2 was precipitated with $BaCl_2$ as $BaCO_3$ and the total amount of CO_2 was determined by titrating the excess NaOH with HCl. The $BaCO_3$ was washed with distilled H_2O, plated (in triplicate) as a slurry on weighed aluminum trays, dried under infrared lamps, and reweighed. The trays were then counted in a methane gas flow counter and the total activity of the respired carbon was calculated.

Results

Table I shows the results obtained for liver, spleen, and kidney slices. When the nonirradiated mice were sacrificed 4 hours after serotonin was administered,

TABLE I

Carbon-14 respired by mouse liver, spleen, and kidney slices

Time (hours)	Liver (% of glucose activity*)		Spleen (% of glucose activity*)		Kidney (% of glucose activity*)	
	Saline	Serotonin	Saline	Serotonin	Saline	Serotonin
Nonirradiated						
4	1.54	4.03	14.81	19.24	20.11	31.00
24	1.77	2.83	14.95	15.07	18.15	20.22
Irradiated						
4	3.14	4.00	19.17	17.72	33.76	32.79
24	2.83	3.38	15.10	16.55	29.41	29.34

NOTE: Each figure represents the average value of three incubated samples and the variation never exceeded ±4% of the average value.

*Each sample was incubated with about 35 μg of C^{14} glucose containing 75×10^3 counts/minute.

there was an increased amount of $C^{14}O_2$ respired by liver, spleen, and kidney slices incubated with C^{14} glucose. Of the tissues examined, liver showed the greatest increase due to serotonin and spleen the smallest. This effect was less marked when the mice were sacrificed 24 hours after the administration of serotonin.

X-Irradiation of the mice 4 hours before they were sacrificed also increased the $C^{14}O_2$ respired by the tissue slices and this effect is in agreement with previous reports (8, 9). Again, the effect of X-irradiation of the mice was greatest on the liver slices and the effect was less marked after 24 hours.

The administration of both serotonin and X-irradiation to the mice increased the $C^{14}O_2$ respired by the liver slices more than did X-irradiation alone. However, this effect of serotonin was not observed for spleen or kidney slices.

Discussion

It is clear that at the times sampled (4 and 24 hours), both serotonin and X-irradiation increase the ability of the tissues to oxidize C^{14} glucose to $C^{14}O_2$. The relation of this to the action of serotonin in the protection against radiation remains unknown. Serotonin must be given about 15 minutes prior to X-irradiation to be effective as a protective agent (2). It may be that the decreasing effectiveness of serotonin as time passes parallels its decreasing effect on tissue oxidation. Experiments are now under way to examine the effects of serotonin at much shorter time intervals after its administration.

The effect of serotonin in stimulating tissue oxidation of glucose at the times noted in the present study is probably related to its effect in stimulating thyroid activity (5).

Acknowledgment

The authors wish to thank S. Faulkner for her valuable technical assistance during this investigation.

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CARDIAC OUTPUT AND RELATED HEMODYNAMIC DATA IN NORMAL CHILDREN AND ADULTS[1]

W. JEGIER, P. SEKELJ, H. T. DAVENPORT, AND M. MCGREGOR[2]

Abstract

Cardiac output, so-called "central blood volume", "slope volume", and time–concentration components of indicator dilution curves were measured in 28 normal children (5–13 years) and 18 normal adults (24–54) years. Determinations were based on dye dilution curves obtained from peripherally injected Evans blue T-1824, using a computing ear oximeter. Average values and ranges of cardiac index in children were 3.19 ± 0.75 (1.92–4.48) liters/minute/sq.meter and 3.06 ± 0.76 (2.04–4.09) liters/minutes/sq.meter in adults. There was no significant difference between the mean values ($P > 0.50$). Close similarity was also observed between the means, standard deviations, and ranges for "central blood volume", "slope volume", and the time–concentration components in children and adults when differences in body size were accounted for. No significant differences were observed between the corresponding values in the two groups. (All P values were greater than 0.25.) Correlations of various experimental data to body surface area are presented.

Normal values for resting cardiac output and time components of indicator curves are now well founded on numerous studies in adults. However, because of the difficulties of making measurements, values for normal children have been less well studied.

The introduction of an automatic computing ear oximeter, from which instantaneous dye concentrations may be read without the need of arterial or venous sampling, has made the measurement of cardiac output a relatively simple procedure (1, 2). This can be carried out without difficulty even in small children.

The present paper describes the values for cardiac output, two central volume measurements, and time–concentration relationships of the indicator curves in 28 normal children. As a basis for comparison the data obtained in 18 normal adults are also reported.

Methods and Material

Twenty-eight normal white children, aged 5–13 years, and 18 normal white adults, aged 24–54 years, were studied. The children were studied under light anaesthesia just before undergoing elective minor surgery. No premedication was employed. In 11 children anaesthesia was induced with 80% nitrous oxide in oxygen through a nonrebreathing circuit and maintained thereafter with 50% nitrous oxide in oxygen. Seventeen children, after induction with 80% nitrous oxide in oxygen, were maintained with 50% nitrous oxide plus 0.25% halothane (Fluothane) delivered by a previously calibrated Rowbotham bottle. After a

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Contribution from the Departments of Cardiology and Anesthesia, The Montreal Children's Hospital, and the Department of Physiology, McGill University, Montreal, Que.
[2]Research Associate of the National Research Council of Canada.

very light plane of anaesthesia was obtained (clinical plane 1 or less) a gauge 19 to 21 needle was introduced into an antecubital vein and attached to a three-way stopcock. A measured amount of 0.5% aqueous solution of Evans blue T-1824 (0.205 to 0.237 mg/kg) was injected rapidly and flushed in with 5 to 10 ml of normal saline.

In the adults the experiments were made without sedation or anaesthesia. The site of venipuncture was infiltrated with 2% procaine and a period of at least 15 minutes allowed to elapse between introduction of the needle and performance of the injection. Room air or 100% oxygen was inhaled. The doses of dye injected and technique of injection were the same as those used in children. Before the injection of dye, in each study, the arterial oxygen saturation was measured with the earpiece and during the inscription of each curve a simultaneous electrocardiogram was recorded.

The time–concentration relationships of the dye dilution curves were analyzed using the definitions of the components given by Swan (3). Appearance time (A.T.), build-up time (B.T.), maximal concentration time (M.C.T.), and disappearance time (D.T.) were measured. The cardiac output (C.O.) was determined according to the method of Hamilton (4). The "central volume" was obtained by the formula of Newman (5) and will be referred to as "slope volume" (S.V.). Values for "central blood volume" were computed by Stewart's formula (6). They will be referred to as "central blood volume" (Q).

Surface areas were obtained from the DuBois chart for adults and older children, and from the DuBois formula for younger children.

Results

Considerable variability of all recorded values within each group and within the groups was observed. An attempt was made to relate the variability encountered in the subjects to body size. The scattergraphs (Figs. 1 and 2) illustrate the relationship of the various data, for both groups, to body surface area. A tendency of the recorded values to increase with body size is evident. Correlation coefficients and regression lines are shown.

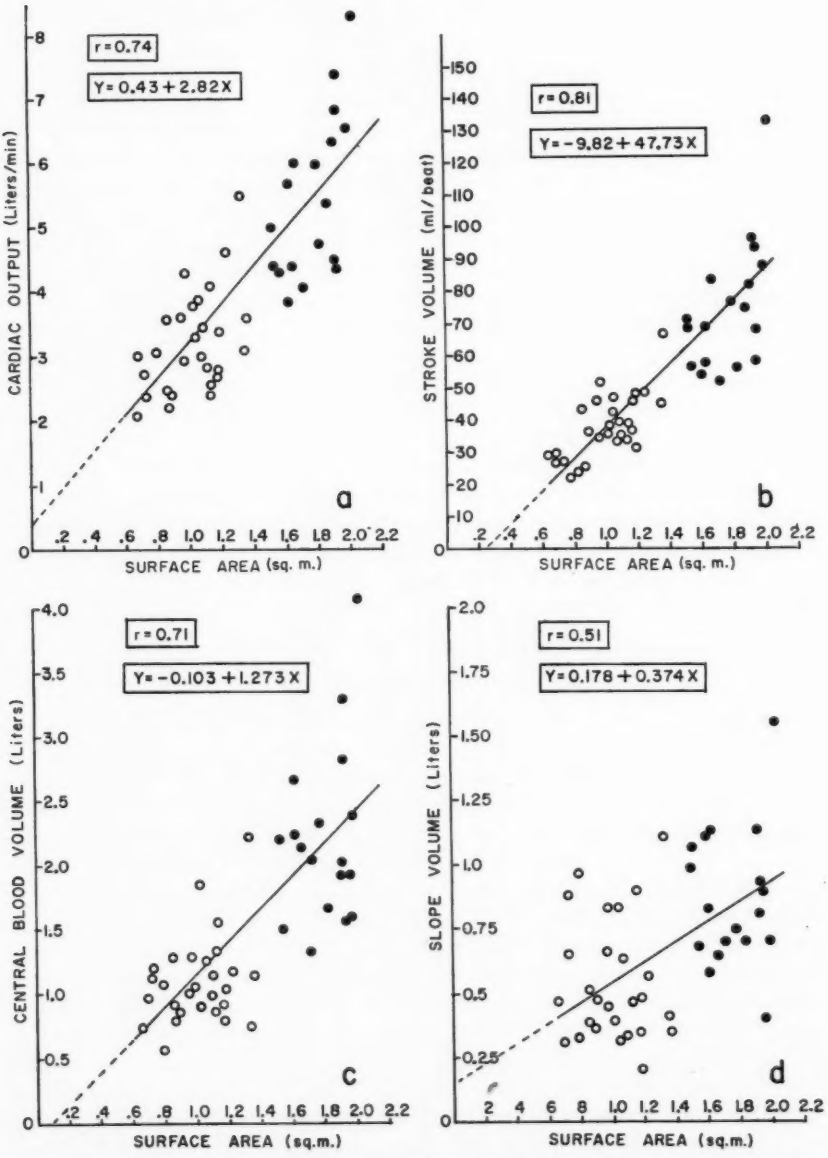
Comparison between the mean data for the two groups (Table I) reveals that in spite of a somewhat higher heart rate and lower stroke index (stroke volume/unit of body surface area) there was no significant difference in cardiac output when related to body size ($P > 0.5$). Likewise when "central blood volume" and "slope volume" were related to body size there were no significant differences between the two groups (P values > 0.5). Comparison of mean values, ranges, and standard deviations of time–concentration measurements made in both groups are shown in Table II(a). This comparison is indicative of a more rapid circulation in the children. However, when the values were related to body size (Table II(b)) it became apparent that there were no statistically significant differences between the two groups (P values > 0.25).

TABLE I

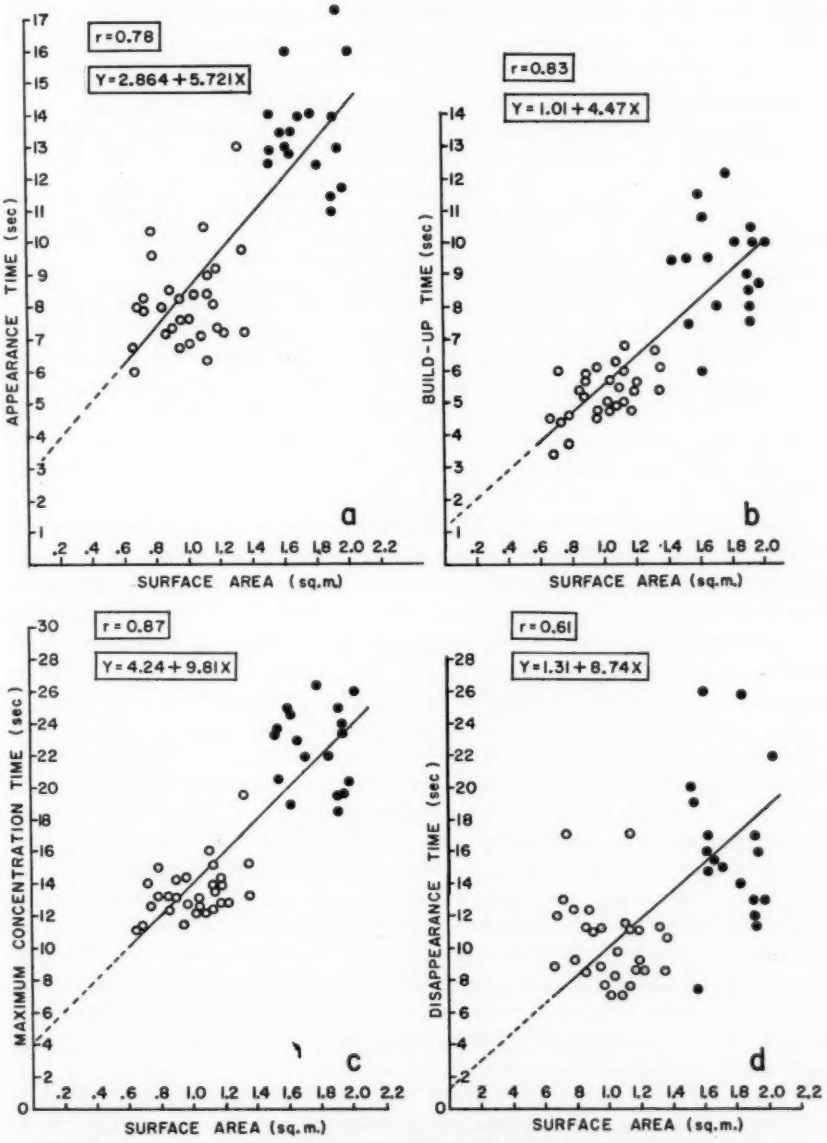
Mean values and ranges of age, surface area, heart rate, cardiac index, stroke index, central blood volume, and slope volume

	Sex (M/F)	Age (years)	Surface area (m^2)	Heart rate per minute	C.I. (l./min/m^2)	S.I.* (ml/beat/m^2)	Q (ml/m^2)	S.V. (ml/m^2)
Children	19/9	9 (5–13)	1.01 (0.67–1.36)	85 (60–108)	3.19±0.75 (1.92–4.48)	36.44±11.23 (22.8–53.8)	1145±359 (558–1770)	510±251 (193–1213)
Adults	11/7	31 (24–54)	1.76 (1.54–2.02)	72 (61–84)	3.06±0.76 (2.04–4.09)	43.36±11.66 (27.9–66.5)	1275±392 (773–2020)	502±176 (207–780)

NOTE: Numbers following ± are the S.D. and numbers in parentheses are the ranges.
*The P value for the differences in this column is less than 0.06.



FIGS. 1*a*, 1*b*, 1*c*, and 1*d*. Scattergraphs showing the relationships of cardiac output, stroke volume, "central blood" volume, and "slope" volume to surface area. Open circles refer to children, solid circles to adults.



FIGS. 2*a*, 2*b*, 2*c*, and 2*d*. Scattergraphs showing the relationships of appearance time, build-up time, maximum concentration time, and disappearance time of dye dilution curves to surface area. Open circles refer to children, solid circles to adults.

TABLE II

(*a*) Concentration–time components

	A.T. (seconds)	B.T. (seconds)	D.T. (seconds)	M.C.T. (seconds)
Children	8.3±1.93 (6.0–13.0)	5.4 ±0.76 (3.4–6.8)	10.07±2.73 (7.0–17.0)	13.74±2.34 (11.3–19.6)
Adults	13.4±1.94 (11.0–17.5)	10.05±2.58 (6.0–12.2)	14.93±4.82 (7.2–26.0)	23.42±3.39 (18.6–26.3)

(*b*) Concentration–time components related to surface area

	A.T./S.A.	B.T./S.A.	D.T./S.A.	M.C.T./S.A.
Children	7.73±1.95 (4.9–13.1)	5.06±0.84 (4.0–8.3)	9.73±4.47 (6.3–23.3)	12.77±2.65 (9.5–19.4)
Adults	7.68±1.41 (5.8–10.3)	5.79±1.76 (3.7–7.20)	8.74±3.24 (4.6–16.3)	13.48±2.66 (9.7–15.7)
P values >	0.50	0.50	0.25	0.60

Discussion

The measurement of cardiac output, in normal children, by whole blood cuvette techniques or by cardiac catheterization presents obvious difficulties and it is presumably for this reason that no data for normal children are available in the literature. The ear oximeter, while making such measurements feasible, is not widely employed and it was therefore thought desirable to study a series of adults, as well, by an identical technique.

The values for adults reported here agree well with those found by other workers using different techniques (7–11). Analysis of our data seems to justify the conclusion that the recognized relationship between cardiac output and body size for adults holds also for children of the size studied here. The observed correlation ($r = 0.74$) between cardiac output and body surface area over the range from about 0.65 to 2.0 square meters agrees reasonably well with that ($r = 0.68$) recently reported by Reeves and associates over a narrower range from about 1.0 to 2.0 square meters (12). There was a better correlation between stroke volume and body surface area ($r = 0.81$) in our study. Further experiment is needed to ascertain whether the linear relationship of cardiac output and stroke volume to body size, which apparently exists over the range of body surface area studied, could be extended into the range of infants and very small children.

Although the "central blood volume" and "slope volume" are not anatomically definable and of doubtful physiological significance, the similarity of these measurements, when related to body surface, is of interest. The "central blood volume", based on determination of the mean circulation time, shows a considerably better correlation to body size than does the "slope volume".

All the time–concentration components of dye dilution curves considered

here, except the disappearance time,* correlate well with body size. High degree of correlation ($r = 0.87$) was found to exist between maximum concentration time and body size.

The question may be asked if the cardiac output measured in subjects under very light anaesthesia with nitrous oxide is comparable with that found in subjects without anaesthesia. The answer to this question is tentatively affirmative. While Fisher *et al.* (13) reported that 80% nitrous oxide in oxygen caused a slight ventricular dilatation in the Starling heart–lung preparation of the dog, they could not determine with certainty that this effect was not due to hypoxia. Price and Helrich (14) also found in the dog's heart–lung preparation some reduction of the myocardial contractility when the heart was exposed to similar concentrations of nitrous oxide. However, Prime and Gray (15) were of the opinion that nitrous oxide in the concentrations used in this study has a negligible effect and Adriani (16) expresses the view that this anaesthetic agent has no effect on the cardiovascular system. Pollock *et al.* (17) found no statistically significant changes in the cardiac output values recorded before and after the induction of anaesthesia with nitrous oxide in oxygen. We did not find any significant difference between the data obtained in the 17 children who were breathing 50% nitrous oxide in oxygen and the 11 others inhaling a mixture of 50% nitrous oxide in oxygen plus 0.25% Fluothane. Anoxia did not occur at any time, the lowest level of arterial oxygen saturation recorded being 95%.

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*It will be noted that the disappearance times reported here were obtained directly from the dye dilution record. Similarly low correlation was obtained when the semilogarithmic replots were used to determine these times.

QUANTITATIVE CHROMATOGRAPHIC METHODS

PART 8. CHROMATOGRAPHIC SYSTEMS OF HIGH RESOLVING POWER FOR NUCLEOTIDES[1]

M. A. MOSCARELLO,[2] B. G. LANE,[3] AND C. S. HANES

Abstract

Two paper chromatographic systems of improved resolving power for nucleotides are described. Both systems are based on buffered miscible solvents, used with chromatographic papers previously impregnated with the same buffers. The potential usefulness of these systems in studies on naturally occurring soluble nucleotides and on degradation products of nucleic acids is illustrated.

Introduction

In earlier communications of this series, paper chromatographic systems having improved resolving power for the amino acids were described (1, 2, 3). These were based on buffered solvents, containing miscible alcohols and aqueous buffer solutions, used with filter papers impregnated separately with the same buffers. Improvement in the chromatography resulted in part from the development of equipment permitting the easy adjustment and control of various factors, such as the rate of irrigation of the chromatogram, and the initial amounts of water and of other volatile liquids present in the system prior to addition of the developing solvent. In parallel with the work on the chromatography of the amino acids, a number of systems of similar type were developed for separating mixtures of soluble nucleotides, such as those which occur in extracts of cells and tissues and in alkaline hydrolyzates of RNA.*

Two such systems developed for nucleotide separations, which will be described in the present communication, employ buffered papers and solvents containing isopropanol, ethanol, and aqueous buffers; a pyrophosphate buffer at pH 7.1 is used in one case, and a tartrate buffer at pH 3.4 in the other. The efficiency of the two systems is shown by their ability to separate ten out of twelve 5′-ribonucleotides applied in a single spot at the origin. In both systems the monophosphates of the ribonucleosides are well separated as a group from the diphosphates, and except for GDP, these are well separated from the triphosphates. The tartrate-buffered system has been used exten-

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Contribution from Department of Biochemistry, University of Toronto, Toronto, Ontario.
[2]Holder of Fellowship of the National Cancer Institute of Canada.
[3]Present address: Rockefeller Institute for Medical Research, New York, N.Y.

*The following abbreviations based in part on those of Markham and Smith (10) and Lane and Butler (4) are used: the symbols a, g, c, t, u denote, respectively, the bases adenine, guanine, cytosine, thymine, and uracil, while A, G, C, and U denote the nucleosides adenosine, guanosine, cytidine, and uridine. A phosphate group is denoted by p and when written to the right, e.g. Ap, it denotes a phosphate attached to the 2′ or 3′ position (of ribose); where a distinction is to be made, the appropriate numeral is inserted, e.g. A2p. When written to the left, e.g. pA, the p denotes a phosphate in the 5′ position; but the common 5′-monophosphates are sometimes denoted by AMP, GMP, CMP, and UMP, in relation to the di- and tri-phosphates which are designated by ADP, GDP, CDP, UDP, ATP, GTP, CTP, and UTP.

RNA refers to ribonucleate and DNA to deoxyribonucleate. Poly A, poly C, and poly U refer to the synthetic polymers of adenylic, cytidylic, and uridylic acids, respectively.

sively for separating the products of alkaline hydrolyzates of RNA and it was found that the mono-, di-, tri-, and tetra-nucleotides form well-separated groups with considerable resolution of the individual components within each group (4, 5). The pyrophosphate-buffered system has been used so far mainly for separating the soluble nucleotides extracted from various tumors and normal tissues. This experience suggests that the two systems will provide useful supplementation of existing procedures for the study of the nucleotides and the nucleic acids.

Materials and Methods

Chromatographic Equipment

The development of the chromatographic equipment has been described earlier (1) and the manner of using the final type 4 chromatographic unit for the separation of the amino acids has been described (3). Studies on nucleotide separations were begun with the type 3 unit with its elaborate preconditioning system. This method of preconditioning was replaced for a time by passing through the unit a stream of conditioned air which had been bubbled through an appropriate solution (cf. 2, p. 132) and results obtained with this method have been described (4, 5). Latterly, as will be seen below, the simpler and more versatile system of preconditioning provided in the type 4 unit has been used.

Marker Solutions

Solutions of the 5′-ribonucleotides were made by dissolving the sodium salts in 0.01 *N* HCl to give a final concentration of 0.02 *M* for each nucleotide. Usually 3 μl of this solution was applied at the starting line. The RNA hydrolyzate was prepared as follows: a 1% solution of RNA in 1 *N* KOH was allowed to stand at room temperature for 2 hours. The solution was cooled and neutralized with cold 12 *N* perchloric acid. The potassium perchlorate was removed by centrifugation and the supernatant was used for spotting on the chromatogram. Hydrolyzates of poly A, poly C, and poly U were prepared in the same manner.

Chemicals

Isopropanol, tetrasodium pyrophosphate decahydrate, and tartaric acid (all of AnalaR grade) were purchased from British Drug Houses. The ethanol was the purest grade 95% (rectified ethanol 65% overproof). The 5′-ribonucleotides were purchased from Sigma Chemical Co. RNA was prepared from yeast by the method of Crestfield, Smith, and Allen (6). Poly A was kindly supplied by Dr. S. Ochoa, New York University, and poly C and poly U were prepared with polynucleotide phosphorylase, which was also the generous gift of Dr. S. Ochoa. Whatman No. 3 HR filter paper, in sheets $29\frac{1}{2} \times 11$ in., was purchased from Reeve Angel and Co.

Preparation of Buffer Solutions

(a) Pyrophosphate Buffer (0.05 *M* pyrophosphate at pH 7.1, 0.34 *M* sodium chloride)

This is prepared as follows: 89.3 g of tetrasodium pyrophosphate decahydrate and 67 g sodium chloride are dissolved in about 3.5 liters of water. The pH is adjusted to 7.1 (glass electrode) by the addition of about 37 ml of 5 *N* HCl and the volume is brought to 4 liters with water.

(b) Tartrate Buffer (0.05 *M* tartrate at pH 3.4, 0.17 *M* sodium chloride)

This is prepared as follows: 30 g of tartaric acid and 40 g of sodium chloride are dissolved in 3900 ml of water and the pH adjusted to 3.4 (glass electrode) by the addition of 10 *N* potassium hydroxide and the volume is then brought to 4 liters with water.

Preparation of Chromatographic Solvents

Solvent 1

Isopropanol – ethanol – aqueous pyrophosphate buffer (25 volumes/40 volumes/35 volumes). Two liters of solvent is prepared by combining 500 ml isopropanol, 800 ml 95% ethanol, and 700 ml of the above pyrophosphate buffer. The mixture is stored at 34° C at which temperature the chromatography is conducted (1). After the mixture has been standing overnight, excess salts will have crystallized as a crust on the bottom and the supernatant solution is decanted as required.

Solvent 2

Isopropanol – ethanol – aqueous tartrate buffer (30 volumes/40 volumes/30 volumes). Two liters of solvent is prepared by combining 600 ml isopropanol, 800 ml 95% ethanol, and 600 ml of the above tartrate buffer. The mixture is stored at 34° C and is used after excess salts have settled out as in solvent 1.

The stated compositions of solvents 1 and 2 were chosen after considerable testing as being nearly optimum for the respective systems. They may be varied within narrow limits, however, to obtain particular results. The content of aqueous buffer in solvent 2, for example, may be varied in the range 27 to 33 volumes % (i.e. $\pm 10\%$) compensated by changes in the content of isopropanol; this results in small (but significant) changes in the pattern of separations, but considerable changes in the rate of migration of the nucleotides as a whole. This point is mentioned because the two systems for nucleotides, especially the tartrate-buffered system, are more sensitive than those developed for the amino acids (3) to variations in the flow characteristics of different batches of chromatographic paper. (This seems to be related to the low R_f values which are necessary in the nucleotide systems to obtain adequate resolution, as compared with the considerably higher range of R_f values of the amino acids.) Accordingly, if the desired rate of migration is not readily attained with a new batch of paper by simple adjustment of the feeder wick, a small change in the solvent composition may be used to adjust the rate; this will be illustrated in chromatograms H and J (Fig. 2).

It should be mentioned that volumes of solvent varying from 275 ml to 425 ml have been added to the trough to irrigate the pair of chromatograms, the volume being varied as a means of finer adjustment of the effective wick length as in the previous work (1, 3).

Preparation of Buffered Chromatographic Papers

The earlier stages of the work were carried out with Whatman No. 3 paper purchased prior to 1957. In order to improve the chromatographic properties and to reduce ninhydrin-reacting and ultraviolet-absorbing impurities, this paper required rigorous purification before use, by a procedure described in Part 1 of this series (7). Later, during the development of the improved new grade No. 3 HR paper, referred to in Part 4 (3, p. 168), the need for this special washing procedure disappeared so far as the chromatography of amino acids and peptides is concerned.

In the case of the nucleotides, however, it was found that the new No. 3 HR paper, as purchased, is much inferior to the old No. 3 paper purified in the laboratory. Following earlier observations on the phosphoric esters (8), a simply preliminary extraction with dilute acetic acid was applied as the first stage in the process of impregnating the batches of paper with buffer solutions. This had the effect of restoring the chromatographic resolution of the nucleotides to an acceptable level, as will be illustrated in Figs. 1–3 in this communication. Accordingly, in the pretreatment of batches of 35 sheets of Whatman No. 3 HR paper with either the pyrophosphate or the tartrate buffer, the first step is to allow the papers to become wetted by the capillary ascent of 0.5 *N* acetic acid whilst they are suspended in the apparatus illustrated in Fig. 6 of Part 2 of this series (1). The upper ends of the papers in which the impurities collect are marked so that this end ultimately forms the bottom of a chromatogram. After the papers have become fully wetted with acetic acid solution (16–20 hours), the pile of papers is transferred to the suction block (1). The acetic acid is then washed out by successively flooding the paper with water, allowing it to stand for 5 minutes, and then drawing off the excess liquid by brief application of suction. After about 10 such cycles, when the washings have become only faintly acid, impregnation of the block with pyrophosphate or tartrate buffer is achieved by the same process, eight cycles being performed, each requiring about 400 ml of buffer solution for the 35 papers. The drying of the buffered papers, and their storage in polyethylene bags, have been described (1).

While this brief acid washing of the paper prior to its impregnation with buffer results in acceptable chromatography, it should be stated that the results remain appreciably inferior to those obtainable by the use of paper which has been subjected to the full purification involving washings with acid – water – lithium hydroxide – water – ethanol which was evolved earlier (7). Such paper is still used in this laboratory for more exacting resolutions.

Assembly of the Chromatogram

As described for the amino acids (1, 3), the assembled chromatogram consists of the main buffered paper, the feeder wick (usually also of Whatman No. 3 HR paper and of a length adjusted to give the desired rate of travel), and the absorption pad, with polyethylene wrapper, clamped to the bottom edge.

Preconditioning of the Closed System

When the pair of chromatograms are placed in position in the type 4 unit, the relative humidity of the atmosphere of the laboratory (with which the papers have been in contact for at least 1 hour) is read from a hair hygrometer (1). This permits the weight of imbibed water in the two chromatograms (x g) to be read from a water content/relative humidity graph for the particular type of buffered paper. Graphs for the pyrophosphate- and tartrate-buffered systems are constructed from the data given in columns 4 and 8, respectively, in Table II of Part 4 of this series (3). (These data relate to similar but not identical buffered papers.) The values given there for the moisture content per 100 g dry paper are corrected to correspond to the weight of two chromatograms of the particular batches of paper used (about 70 g).

For the preconditioning of the pyrophosphate-buffered system, the closed unit, prior to addition of solvent, should contain a total of 7 ml water and 7 ml isopropanol. Accordingly, $(7-x)$ ml water and 7 ml isopropanol are added to the external receiver tube which leads to the evaporator inside the unit. The motor-driven fan is turned on for a period of 30 minutes, after which the required volume of solvent 1 is added to the trough. In the case of the tartrate-buffered system the preconditioning is carried out with 7 g water and 10 ml isopropanol, the procedure being the same.

The Ammonium Sulphate System of Markham and Smith

The chromatographic system described by Markham and Smith (10) produces a very different pattern of separation of the nucleotides which makes it a useful complement for the new systems which have been described. The solvent is made up of 79 volumes saturated aqueous ammonium sulphate solution, 19 volumes 0.1 *M* sodium acetate buffer of pH 6.0, and 2 volumes isopropanol. It has been used effectively to resolve certain components of alkaline hydrolyzates of RNA after preliminary separation into groups on the tartrate-buffered system.

For use in the type 4 unit, the standard sheets of No. 3 HR paper, without buffer, are provided with a feeder wick of No. 1 Whatman paper (1). For best results, the pair of papers should contain no more than 2 to 3 g imbibed water (corresponding to equilibrium with a relative humidity of ca. 30%). It may be found advantageous under moist atmospheric conditions to remove excess water from the system by the use of weighed sacs of silica gel as described in Part 4 (3).

Results

Resolutions in Pyrophosphate-Buffered System

In Fig. 1 is shown a selection of chromatograms developed in solvent 1 on the pyrophosphate-buffered paper.

The effects of variation in the rate of supply of solvent on the resolution of the 5′-ribonucleotides are illustrated in chromatograms C, D, E, and F. The

wick in C was of the thin Whatman No. 1 paper of 31 mm effective wick length (i.e. distance from surface of the liquid to the line of stitching). Although the total movement was restricted (pU travelling only about 12 inches) the spots are compact and the separations of the monophosphates good. In chromatogram F the wick was of No. 3 HR paper also of 31 mm effective length and this produced an excessive rate of migration; pU was lost from the bottom, and ATP, UTP, and GDP failed to separate. By increasing the wick of No. 3 paper to 43 mm effective length, chromatogram E was obtained, which shows approximately the optimum resolution obtainable with this system. With a slow-flowing specimen of No. 3 HR paper, used in chromatogram D, the migration in 40 hours was slightly less than optimum with some loss of resolution (e.g. UDP) even though a very short wick of No. 3 paper (17 mm effective length) had been used. In this case a longer irrigation time or a slight adjustment of the solvent composition (e.g. increasing the aqueous buffer to 37 volumes % at the expense of isopropanol) or an increase in the initial water content for preconditioning from 7 to (say) 9 g might be advantageous.

The 2′- and 3′-monophosphates of the different ribonucleosides are not separated in this system although incipient separations are seen in chromatograms A and B. A2p migrates a little faster than A3p and, in B, the chromatogram of an alkaline hydrolyzate of RNA, the beginnings of resolution of U2p and U3p and, to a lesser extent, of G2p and G3p may be seen.

It is evident that the mononucleotides as a group are well separated from the dinucleotides; and the tri- and tetra-nucleotides also are cleanly separated.

Chromatogram G shows the resolution of the homologous series of derivatives of adenine, guanine, cytosine, and uracil, respectively, obtained by development of the chromatogram for only 24 hours. In all series the three phosphorylated derivatives are cleanly separated; the bases are separated from the nucleosides in the cases of adenine and guanine while uracil remains contiguous with uridine, and cytosine and cytidine migrate as one component. The four bases applied as a single spot are resolved into three spots only, adenine and uracil migrating together.

Resolutions in Tartrate-Buffered System

In Fig. 2 is shown a selection of chromatograms developed in solvent 2.

The resolution of the 5′-ribonucleotides is shown in chromatogram J; as in the pyrophosphate-buffered system, UTP and GDP migrate together, so that 11 spots are discernible but pC and pA are contiguous.

The migration of the components of the RNA hydrolyzate in J was excessive. This was adjusted satisfactorily in H by decreasing slightly (to 27 volumes %)

FIG. 1. The pyrophosphate-buffered system.
Chromatograms A–F irrigated for 40 hours and G for 24 hours with solvent 1. For abbreviations see footnote, p. 1755. Chromatogram B shows the products of alkaline hydrolysis of RNA. Wicks used: chromatogram C, Whatman No. 1 paper, 31 mm effective length; chromatogram E, Whatman No. 3 paper, 31 mm effective length; other chromatograms, Whatman No. 3 paper, 43 mm effective length. All chromatograms were run on Whatman No. 3 HR paper but that used in D was from a slow-flowing batch.

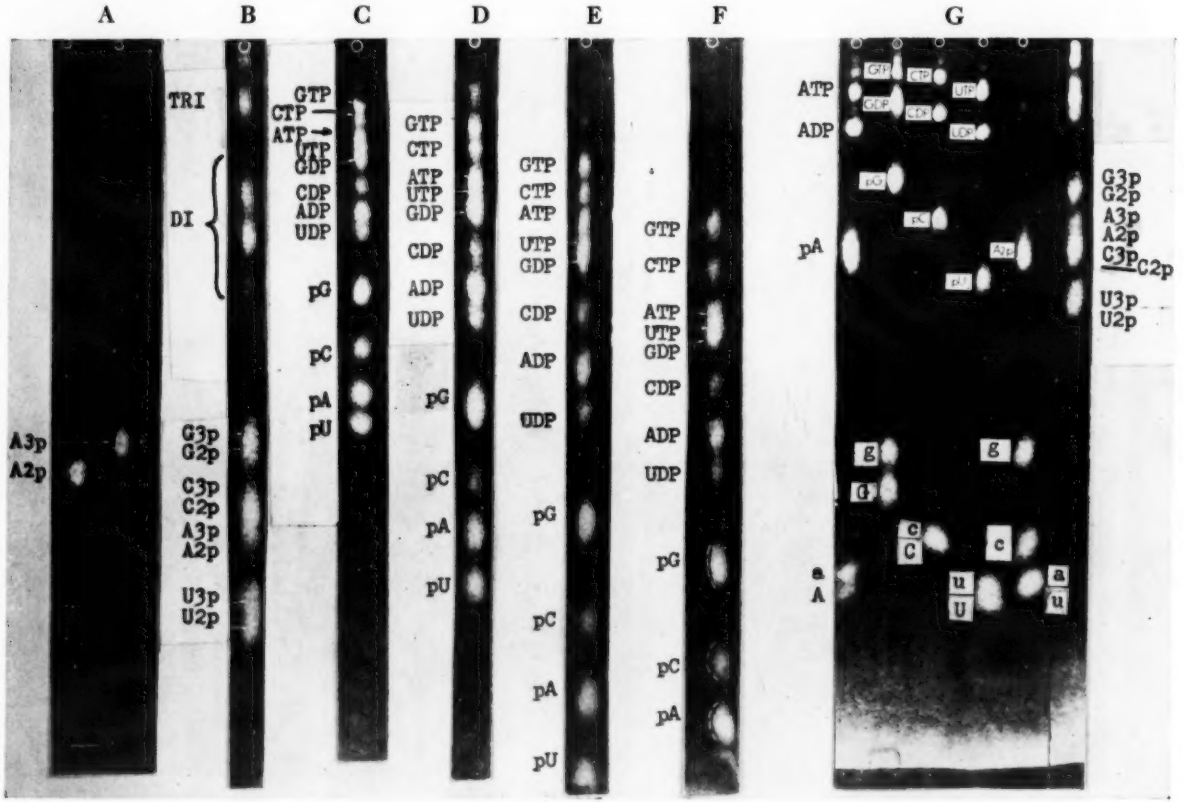

FIG. 1.

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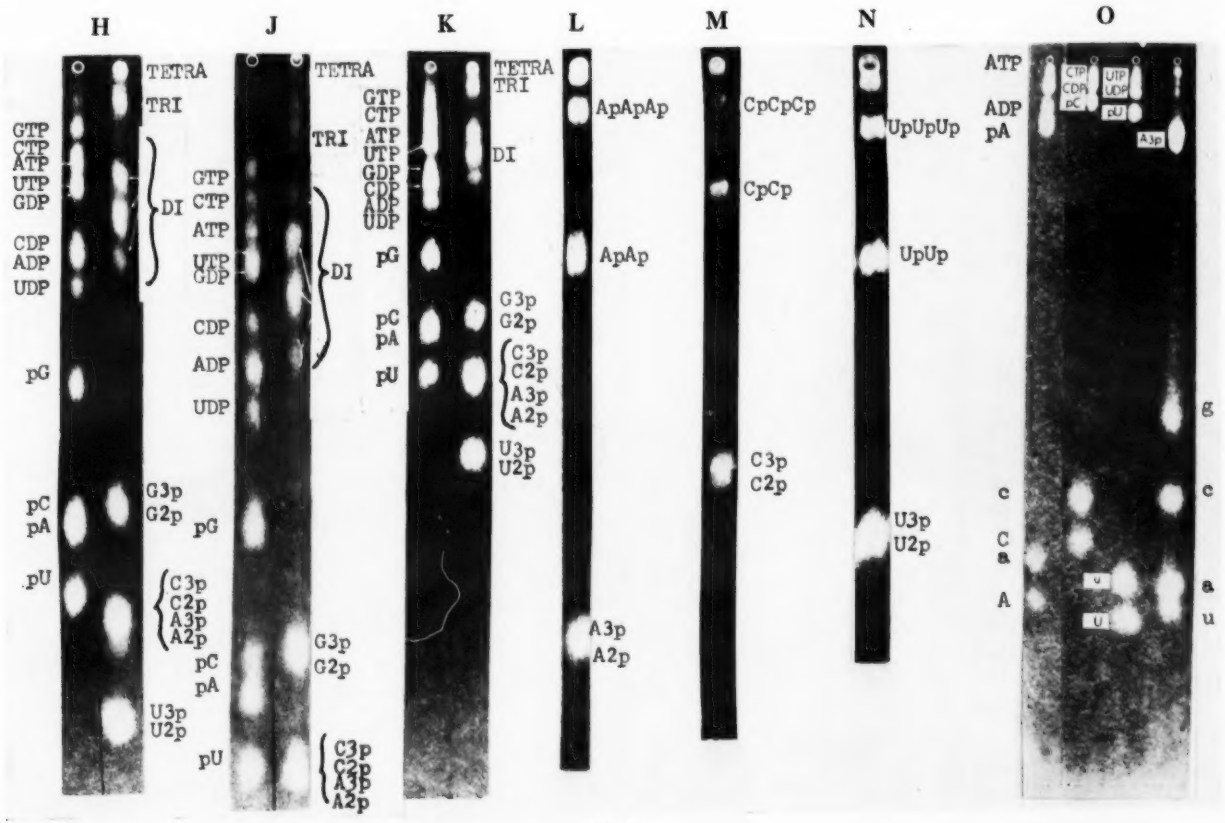
H
J
K
L
M
N
O
TETRA
TRI
DI
GTP
CTP
ATP
UTP
GDP
CDP
ADP
UDP
pG
pC
pA
pU
G3p
G2p
C3p
C2p
A3p
A2p
U3p
U2p
ApApAp
ApAp
CpCpCp
CpCp
UpUpUp
UpUp
ATP
ADP
pA
g
c
C
a
A
u

FIG. 2.

the content of aqueous tartrate buffer in the solvent, as was mentioned above, U3p and U2p being then retained on the chromatogram. It should be noted that the separations of some of the 5′-ribonucleotides were impaired by this change in solvent composition.

The effect on the migration rate, in chromatogram K, of using the slow-flowing specimen of No. 3 HR paper (illustrated previously in Fig. 1D) is very marked in the tartrate-buffered system; as in H, the wick was of No. 3 HR paper of 43 mm effective length.

The resolutions of the components of the alkaline hydrolyzates of poly A, poly C, and poly U are shown in chromatograms L, M, N respectively. In all cases good separations are obtained of the tri-, di-, and mono-nucleotides. It should be mentioned that these three chromatograms were irrigated for different lengths of time. Consequently the distances of migration are not comparable for the different chromatograms.

Chromatogram O shows the resolution of the different homologous series (cf. chromatogram 6, Fig. 1). It is evident that the members of the different series are not as well separated in the tartrate-buffered as in the pyrophosphate-buffered systems, but the four bases are better resolved in O.

Resolutions in the Ammonium Sulphate System

An unusual feature of the patterns of separations obtained in the ammonium sulphate system, illustrated in chromatogram Q (Fig. 3), is that the di- and tri-phosphates of each of the four ribonucleosides migrate slightly faster than the homologous monophosphate (the latter, in turn, migrates slightly faster than the free base, although not shown in Fig. 3). It is clear that the nature of the base is the main determinant of the migration rate of each homologous series.

The usefulness of this system for the study of the alkaline degradation products of RNA may be judged from chromatograms P, Q, and R. In P, for example, one of the dinucleotide fractions isolated as a band from a preparative tartrate-buffered chromatogram (i.e. a band which corresponded to the middle dinucleotide spot of chromatogram H of Fig. 2) was rechromatographed in the ammonium sulphate system. The major components separated in P were identified as ApAp, ApCp, and GpUp; there are present also two faint bands, each consisting of mixtures of dinucleotides (4, 5).

Chromatogram R shows the separation of A3p, A2p, G3p, and G2p as discrete spots, from a mixture of eight 2′- and 3′-isomers. It seems possible that

FIG. 2. The tartrate-buffered system.

Chromatograms H–N were irrigated for 40 hours and O for 24 hours with solvent 2 (for chromatogram H, the solvent with slightly reduced water was used, cf. p. 1760, but the normal solvent for all others). For abbreviations see footnote, p. 1755; right-hand rows of chromatograms H, J, and K show products of alkaline hydrolysis of RNA, and chromatograms L, M, and N show the products of alkaline hydrolysis of poly A, poly C, and poly U, respectively. Wicks used: Whatman No. 3 paper throughout, all of effective length approximately 31 mm except chromatograms H and K, which were 43 mm. All chromatograms were run on Whatman No. 3 HR paper, but that used in chromatogram K was of a slow-flowing batch.

some combination of this system and the pyrophosphate-buffered system might provide a basis for resolving the remaining isomers of this group.

Finally, in chromatogram Q, the separation of the components of the alkaline hydrolyzate of RNA is highly distinctive and shows good resolution of this complex mixture. It seems likely that some combination of this system and the pyrophosphate-buffered system (as illustrated in B of Fig. 1, for example) will prove useful either by the use of a two-dimensional technique or by sequential one-dimensional separations.

Discussion

The two isopropanol – ethanol – aqueous buffer systems (buffered at pH 7.1 with pyrophosphate and at pH 3.4 with tartrate, respectively) yield basically similar patterns of separation of the nucleotide compounds and constituents which have been studied. In both systems, under optimum conditions, ten out of twelve 5′-ribonucleotides can be separated in the order (which is the same in both systems), from slowest to fastest: GTP, CTP, ATP, (UTP+GDP), CDP, ADP, UDP, pG, pC, pA, and pU.

The two systems yield also a similar pattern of separations of the products of alkaline hydrolysis of RNA. The mononucleotides, in three discrete spots, are readily separated as a group from the dinucleotides. The dinucleotides, grouped in four spots,* are separated cleanly from the trinucleotides, and the latter are probably separated as a group from the tetra- and higher oligonucleotides. The only detailed study of those separations is that described by Lane and Butler (4, 5) using the tartrate-buffered system. The three mononucleotide spots are identified as Gp, Cp+Ap, and Up, the 2′- and 3′-isomers being inseparable in all cases. The four dinucleotide spots were identified as GpGp, ApGp+GpAp+GpCp, ApAp+ApCp+GpUp, and ApUp. Other dinucleotides, present in trace amounts only, all move in the region bounded by GpGp and ApUp, with the exception of UpUp which migrates faster than ApUp but well behind Gp. The trinucleotides appear to be separated completely as a group from the dinucleotides, except that the fastest-running trinucleotide, UpUpUp, overlaps the slowest-running dinucleotide, GpGp. This presents no difficulty in practice since UpUpUp is not present in significant amounts in alkaline hydrolyzates of RNA.

In view of the potential importance of such resolutions for constitutional studies of RNA, it seems desirable that similar detailed studies should be made of the separations obtained in the pyrophosphate-buffered system, which appears in general to be chromatographically superior to the tartrate-buffered system. It should be mentioned, too, that these complex separations are likely to be of the most exacting and hence are likely to benefit by use of highly purified chromatographic paper to which reference has been made earlier.

*The dinucleotide GpGp is present often in low amount. It can be seen faintly in Fig. 2 as the slowest-moving dinucleotide, and is more evident when the fully purified paper is used (4, 5).

PLATE III

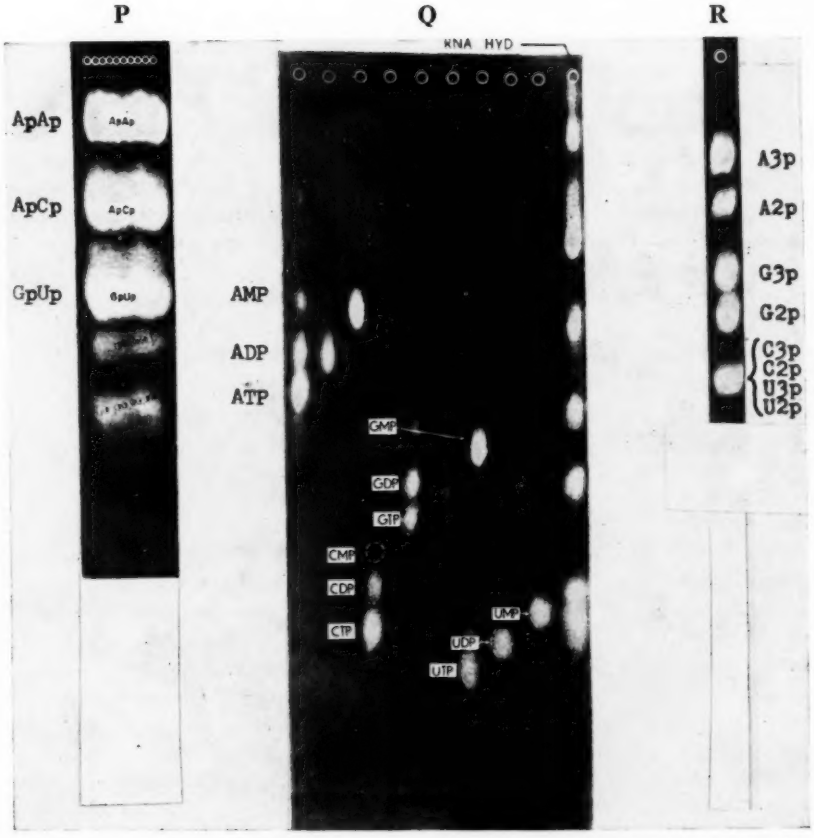

FIG. 3. The ammonium sulphate system of Markham and Smith (10) as described in the text. (P) Resolution of component "X" of the dinucleotide fraction isolated from a preparative chromatogram (4). (Q) Separations of nucleoside phosphates and RNA hydrolyzate. (R) Resolution of the components of the mononucleotide fraction of alkaline hydrolyzates of RNA.

Many of the separations obtained in the two buffered miscible alcohol systems are obtainable also by the use of other systems which have been described by earlier workers, e.g. Carter (11) and Magasanik *et al.* (12), but no single system, except possibly that of Palladini and Leloir (13), seems to provide so generally advantageous a pattern of separations as either of the new systems. Mention has been made above of the very different pattern of separations given by the ammonium sulphate system of Markham and Smith, which is unusual in that the solvent consists essentially of an aqueous salt solution.

It is of interest to consider finally the contrasting chromatographic behavior of the compounds under study in the two types of system. The rates of migration of the individual members of the different homologous series will be governed by the nature of the purine or pyrimidine base, by the presence of the neutral pentose residue (and by the hydroxylation or non-hydroxylation of the residue at the C2 position), by the number (and position) of phosphoryl groups and hence the number of negative charges, and, finally, by the molecular size (reflecting the degree of polymerization).

It is clear that, in the two buffered alcoholic systems, the major determinants of the migration rate are the number of phosphoryl groups and the molecular size. Thus, although the free bases run in the distinctive order (from slowest to fastest) g, c, a, u, and t, and the corresponding derivatives of each run in the same order (e.g. G, C, A, U, and T or pG, pC, pA, pU, and pT), yet the dominant (retarding) effect of the degree of phosphorylation is shown by the fact that the monophosphates, diphosphates, and triphosphates of the nucleosides, irrespective of the nature of the bases, are separated as groups from one another. Similarly, the dominance of molecular size as a factor is shown by the separation of mononucleotides, dinucleotides, trinucleotides, and higher polymers as groups from one another.

In the aqueous ammonium sulphate system, on the other hand, it is evident that the nature of the purine or pyrimidine base and the molecular size are the main determinants of the migration rate and that the presence of phosphoryl groups exerts only a slight (accelerating) effect. The characteristic order of migration of the different bases (and of their homologous derivatives) in this system is a, g, c, and u (compared with g, c, a, and u in the buffered alcoholic systems).

A similar analysis of the factors affecting the patterns of separation of nucleotides and related compounds in other chromatographic systems may well prove useful at this stage when the recent discovery of various new nucleotides places additional demands upon means of identification. Although the survey which has been described in the present communication is far from complete it is sufficient to suggest that systematic knowledge of the factors governing the relative rates of migration of these compounds of great current interest, will make possible the refinement of existing systems and the development of new systems to meet the new requirements.

Acknowledgments

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STUDIES ON THE IN VITRO ANTITUMOR ACTIVITY OF FATTY ACIDS

IV. THE ESTERS OF ACIDS CLOSELY RELATED TO 10-HYDROXY-2-DECENOIC ACID FROM ROYAL JELLY AGAINST TRANSPLANTABLE MOUSE LEUKEMIA[1]

GORDON F. TOWNSEND, WILLIAM H. BROWN, ETHEL E. FELAUER, AND BARBARA HAZLETT

Abstract

Previous studies have shown that whole royal jelly, a fraction from royal jelly (10-hydroxy-2-decenoic acid), and certain closely related dicarboxylic acids, some of which are also found in royal jelly, will inhibit the development of transplantable AKR leukemia when the pH is below 5.6.

The ester of 10-hydroxy-2-decenoic acid from royal jelly was found to be just as effective against AKR leukemic cells as the acid itself, with the added advantage that it could be used at neutrality.

Through the testing of a series of mono- and di-carboxylic acids, as well as other closely related compounds, the activity has been shown to be associated mainly with 9- and 10-carbon straight chain monocarboxylic acids either saturated or unsaturated. Slight variations in the structure either reduce or destroy the activity.

Introduction

Previous studies (1, 2) have shown that whole royal jelly or a fraction from royal jelly (10-hydroxy-2-decenoic acid) and certain closely related dicarboxylic acids (3), as well as monocarboxylic acids (4), some of which are also found in royal jelly (5), will inhibit the development of transplantable AKR leukemia when the pH is below 5.6.

Preliminary tests indicated that the esters of 10-hydroxy-2-decenoic acid are just as effective against AKR leukemic cells when mixed in vitro at a neutral pH as is the acid itself at a pH below 5.6.

In an attempt to determine the relationship of the chemical structure of the fatty acids and their antitumor activity the investigation was extended to the esters of a series of mono- and di-carboxylic acids, as well as some of the structures closely related to 10-hydroxy-2-decenoic acid. The present paper reports these results.

Materials and Methods

An early method of Burchenal and associates (6), using male mice 5–6 weeks of age of the AKR strain,* was used. The spleen of a mouse dying of trans-

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Contribution from Departments of Apiculture and Chemistry, Ontario Agricultural College, Guelph, Ontario, and Department of Therapeutics, University of Toronto, Toronto, Ontario.

*The AKR mice used in these experiments were purchased from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, U.S.A.

planted leukemia was homogenized with a ground-glass homogenizer. Cells were suspended in Gey's balanced salt solution (7) modified by using K_2HPO_4 in place of KH_2PO_4 in order to keep the pH above 7.0 when the cells were added. The suspension was then filtered by gentle suction through cotton and diluted to approximately 30 million cells per ml of cell suspension. The first transfer was made from a mouse dying from spontaneous leukemia, and successive transfers were made as the mice showed signs of dying from leukemia. All of the tests reported in this paper were made with cells carried by successive transfers taken from one original mouse dying from spontaneous leukemia.

The esters to be tested were suspended in Gey's solution with a 2% weight per volume concentration of Tween 80. The addition of Tween 80 had no effect on the survival of the control mice. Appropriate dilutions were made to give double the concentration required in 1 ml.

One milliliter of cell suspension was added to 1 ml of the suspended ester, shaken, and let stand for 5 minutes before subcutaneous injection into the right side of an AKR mouse at a dose of 0.2 ml per mouse. Thus each mouse received 3 million cells as measured by direct count in a hemocytometer. The pH was measured with a Beckman pH meter just before inoculation and was found to range between 7.0 to 7.4. Control mice were treated in exactly the same manner, except for the omission of the ester.

The criterion used in these experiments was survival. The mice either developed leukemia or were protected. Each dead mouse was examined grossly. Sections were prepared from liver and spleen when the gross examination did not reveal leukemia. The experiments were terminated at 90 days in order to distinguish between transplanted leukemia and spontaneous leukemia.

The esters used were all ethyl esters prepared by treatment of the acid with either diazoethane or ethanol and dry hydrogen chloride. Polymerized 10-hydroxy-2-decenoic acid was prepared by heating the monomeric acid at 160–165° C for 8 hours. The α,β-unsaturated acids, 2-nonenoic and 2-decenoic, were prepared by condensation of the appropriate aldehyde with malonic acid according to the method of Brown *et al.* (8). Amides were prepared by treatment of the acid chlorides with aqueous ammonia solution and nitriles were made from the corresponding amide by dehydration with P_2O_5 (9).

Results

In Vitro Antileukemic Activity of Esters of Dicarboxylic Acids

Graded amounts from 2.5 mmoles to 10 mmoles per ml of cell suspension of saturated ethyl esters of dicarboxylic acids in a series from glutarate to tetradecanedioate (C_5 to C_{14}) were mixed with cell suspensions of transplantable AKR leukemia. Typical results of these experiments are summarized in Table I.

It is evident that all of the esters of C_7 to C_{14} acids showed activity, but the greatest activity was exhibited with compounds having carbon numbers from

TABLE I

Activity of ethyl esters of dicarboxylic acids against transplantable AKR leukemia

Acid	Concn. in mmoles	Concn. in mg/ml	Average survival (days)		Survivors at 90 days
			Test	Control	
Pentanedioic	5.0	0.94	17	15	0/7
(Glutaric)	10.0	1.88	17	14	0/7
Hexanedioic	5.0	1.01	18	15	0/7
(Adipic)	10.0	2.02	15	14	0/6
Heptanedioic	5.0	1.08	18	15	0/7
(Pimelic)	10.0	2.16	∞	14	5/5
Octanedioic	2.5	0.57	17	14	0/7
(Suberic)	5.0	1.15	24	15	6/7
	10.0	2.30	∞	14	5/5
Nonanedioic	2.5	0.61	19	13	8/9
(Azelaic)	5.0	1.22	41	15	5/7
Decanedioic	2.5	0.64	29	14	4/9
(Sebacic)	5.0	1.29	∞	18	16/16
Undecanedioic	2.5	0.68	16	9	3/5
(Hendecanedioic)	5.0	1.36	16	14	10/11
Dodecanedioic	5.0	1.43	20	15	3/6
	10.0	2.86	∞	15	4/4
Tridecanedioic	5.0	1.50	17	12	0/5
Tetradecanedioic	2.5	0.79	16	12	1/5
	5.0	1.57	17	12	2/5

NOTE: ∞ All mice survived beyond 90 days.

9 to 11. No activity could be demonstrated below a concentration of 2.5 mmoles.

In Vitro Antileukemic Activity of Esters of Monocarboxylic Acids

Graded amounts from 0.25 mmole to 10 mmoles per ml of cell suspension of saturated ethyl esters of monocarboxylic acids in a series from valerate to tetradecanoate (C_5 to C_{14}) were mixed with cell suspensions of transplantable AKR leukemia. Typical results of the experiment are summarized in Table II. All the esters showed activity from C_6 to C_{13}. The greatest activity was exhibited in esters of monocarboxylic acids of a chain length C_8 to C_{10}.

Activity of Compounds Closely Related to 10-Hydroxy-2-decenoic Acid

Various compounds, particularly those of 9- and 10-carbon structure, were tested at different strengths. The results are summarized in Table III. Slight changes in the structure such as the introduction of a keto or amide group either considerably reduced or eliminated the activity. There appears to be little difference between the saturated or unsaturated acids and polymerization completely nullifies the activity. The ethyl linoleate and ethyl oleate were included since on oxidative fission of the molecule *in situ* there could arise moieties of 9-carbon chain length.

TABLE II

Activity of ethyl esters of monocarboxylic acids against transplantable AKR leukemia

Acid	Concn. in mmoles	Concn. in mg/ml	Average survival (days)		Survivors at 90 days
			Test	Control	
Pentanoic	5.0	0.65	15	15	0/8
(Valeric)	10.0	1.30	16	14	0/7
Hexanoic	5.0	0.72	19	15	0/8
(Caproic)	10.0	1.44	∞	14	7/7
Heptanoic	5.0	0.79	16	15	0/10
(Enanthic)	10.0	1.58	45	14	5/6
Octanoic	2.5	0.43	13	13	0/5
(Caprylic)	5.0	0.86	∞	15	8/8
Nonanoic	0.5	0.09	11	13	0/9
(Pelargonic)	1.0	0.18	∞	11	10/10
	2.5	0.46	∞	14	9/9
	5.0	0.93	∞	15	7/7
Decanoic	0.5	0.10	∞	13	5/5
(Capric)	1.0	0.20	∞	13	5/5
	2.5	0.50	∞	14	5/5
	5.0	1.00	∞	19	17/17
Undecanoic	2.5	0.53	14	14	0/6
(Hendecanoic)	5.0	1.07	∞	15	8/8
Dodecanoic	1.0	0.23	18	9	0/5
(Lauric)	2.5	0.57	23	14	3/5
	5.0	1.14	∞	15	6/6
Tridecanoic	2.5	0.60	17	12	0/5
	5.0	1.21	16	14	4/5
Tetradecanoic	5.0	1.28	17	14	0/5
(Myristic)	10.0	2.56	14	13	0/5

NOTE: ∞ All mice survived beyond 90 days.

Discussion

The observation that the ingredient in royal jelly active in vitro against a number of experimental mouse tumors is 10-hydroxy-2-decenoic acid (1, 2) made it of interest to determine the structure required to give such activity. The present results have shown that varying activity is found in both mono- and di-carboxylic acids from C_6 to C_{14}. The greatest activity, however, is found in the monocarboxylic acids, particularly decanoic acid. There is little if any difference between the activity of the esters of saturated and unsaturated 10-carbon compounds. The polymerized acid is inactive, as are the amides. It is interesting to note that the ethyl ester of 9-keto-2-decenoic acid is inactive. This material in acid form is secreted by the mandibular glands of the queen honey bee (10), and 10-hydroxy-2-decenoic acid is secreted by the mandibular glands of the worker honey bee (11).

Since little attention has been paid to fatty acids as antitumor agents, it is of interest to find that the ester of a simple relatively non-toxic 10-carbon acid such as decanoic should demonstrate in vitro antileukemic activity in such a low concentration as 0.1 mg per ml of cell suspension.

TABLE III

Activity of compounds closely related to 10-hydroxy-2-decenoic acid

Material	Concn. in mmoles	Concn. in mg/ml	Average survival (days)		Survivors at 90 days
			Test	Control	
Ethyl 10-hydroxy-2-decenoate	1.25	0.26	10	14	0/5
	2.50	0.53	∞	14	5/5
	5.00	1.07	∞	21	8/8
Ethyl 10-hydroxy-decanoate	1.25	0.27	18	14	0/5
	2.50	0.54	∞	14	5/5
	5.00	1.08	∞	21	10/10
Polymerized 10-hydroxy-2-decenoic acid	5.00	0.84	24	21	0/10
	10.00	2.00	13	13	0/5
Ethyl 2-nonenoate	0.25	0.05	13	12	0/4
	0.50	0.09	∞	13	4/4
	1.00	0.18	∞	13	5/5
Ethyl 2-decenoate	0.25	0.05	14	12	0/5
	1.00	0.20	—	13	5/5
	2.50	0.49	∞	15	5/5
	5.00	0.99	∞	15	5/5
9-Keto-2-decenoic acid (queen substance)	1.00	0.21	12	13	0/5
	2.00	0.42	12	13	0/5
	4.00	0.85	19	12	0/4
2-Decenonitrile	0.50	0.07	14	13	0/5
	1.00	0.15	24	13	3/10
	1.25	0.19	∞	14	5/5
	2.00	0.30	∞	13	4/4
Decanonitrile	1.25	0.19	13	14	0/5
	2.00	0.30	∞	13	4/4
	5.00	0.76	∞	15	4/4
Decanamide (Capramide)	5.00	0.85	14	15	0/5
10-Hydroxy-2-decenamide	5.00	0.92	14	15	0/5
Ethyl linoleate	5.00	1.54	16	15	0/5
Ethyl oleate	5.00	1.55	15	15	0/5
Nonanonitrile	0.50	0.07	11	9	0/5
	1.00	0.14	11	9	0/5
Sebaconitrile	1.00	0.16	10	9	0/5
Ethyl sebaconitrile	1.00	0.21	13	9	0/5

NOTE: ∞ All mice survived beyond 90 days.

The fact that the ester forms are active at neutrality against AKR leukemic cells makes it much simpler to now make further trials in vivo.

Acknowledgments

We wish to acknowledge the technical assistance of Mr. Ivan Kubovica in the completion of the experiments.

We wish to express our appreciation to the Department of Pathology, Ontario Veterinary College, Guelph, for preparation of the liver and spleen sections.

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ZONE ELECTROPHORESIS OF ACID MUCOPOLYSACCHARIDES[1]

T. S. FOSTER[2] AND R. H. PEARCE[3]

Abstract

Sensitive stains for acid mucopolysaccharides on filter paper have been devised based on the use of either methylene blue or Alcian blue. The electrophoretic migration of hyaluronate, chondroitin-4-sulphate, and heparin in paper followed the Helmholtz–Smoluchowski law for times up to 16 hours and potential gradients up to 3 volts per cm. Under these conditions, the mobility depended upon the ionic strength and component ions of the electrolyte, the width of the strip, and the volume, concentration, and degree of polymerization of the acid mucopolysaccharide. Good separations of mixtures of acid mucopolysaccharides have been achieved, using lithium sulphate or lithium acetate – acetic acid buffer of ionic strength 0.10, on paper strips and rubber sponges as well as by continuous flow electrophoresis. Analysis of the paper strip may provide a sensitive test of the homogeneity of the polysaccharide.

Efficient investigation of the physiology and metabolism of the connective tissues requires convenient methods for the separation of mixtures of the acid mucopolysaccharides. Workers in this field have used fractional precipitation of the calcium salts with ethanol (1), chromatography on paper or columns (2, 3, 4, 5, 6, 7), and fractionation of cetylpyridinium salts with graded concentrations of electrolytes (8, 9). Since the major groups of acid mucopolysaccharides differ in the number of carboxyl and ester sulphate groups attached to the repeating unit of the polymer, zone electrophoresis has also been employed using, as a supporting medium, celite (10) and paper (11–26). A variety of stains has been employed to locate the polysaccharides on paper: toluidine blue (12, 27, 28, 29, 30), Alcian blue (31, 32), mucicarmine (29, 32), colloidal iron (31, 33), cetavlon – bromcresol purple (34), and albumin–Amidoschwartz (35).

Despite this, no attempt seems to have been made to investigate systematically the conditions optimal for separation. Good conditions and a sensitive stain for the common acid mucopolysaccharides were devised in this laboratory a number of years ago. With a few minor modifications, this procedure has been utilized extensively and with uniform success for several methods of zone electrophoresis. The sample required has been smaller and the separations better than have been published elsewhere. This procedure is reported with the hope that it will be useful to other scientists in this field.

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[2]Present address: Animal Research Institute, Research Branch, Canada Department of Agriculture, Ottawa, Ontario.
[3]Present address: Department of Pathology, Faculty of Medicine, University of British Columbia, Vancouver, B.C.

Methods

Apparatus

The tank used for the development of this method was essentially that of Flynn and de Mayo (36), except that the buffer compartments were connected by slits rather than wicks, platinum wire rather than carbon electrodes was used, and the electrodes were placed in the inner compartments, the paper in the outer, not the reverse, in order to make the apparatus compact. For measurement of the potential gradient in the paper, Nichrome wire was threaded through the strips of paper 5 cm from each end; the wire was extended up the side of the tank and under the rim, where it was attached to a Simpson Volt-Ohm-Ammeter, Model No. 160.

During recent years, another apparatus in which the paper was supported horizontally has been found to be convenient. A rectangular Plexiglass tank was used, the lower half of which was divided into two separate, adjacent compartments. Each of these was subdivided by two partitions pierced with many fine slits. Platinum wire electrodes were placed in the inner pair of subcompartments, and the paper dipped in the outer pair. The paper was supported horizontally on unbraided nylon fishing line strung at 1-cm intervals across a Plexiglass rack which, in turn, rested on top of the electrolyte compartments. The tank was sealed with a $\frac{1}{2}$-in. sheet of plate glass rimmed with sponge rubber, which rested on the upper rim of the tank.

Materials

Hyaluronate was prepared from human umbilical cords (37), and chondroitin-4-sulphate from bovine tracheas (38). The heparin used was the sodium salt prepared by Connaught Laboratories.

Electrophoresis

The electrolyte compartments of the tank were filled and levelled by siphoning with a U-tube. The paper was cut into strips, usually 3×70 cm of Munktell No. 20 (150 g), for the horizontal type. The line of application was marked with a pencil, the paper was moistened in the electrolyte, blotted, placed in the apparatus, and allowed to equilibrate for at least 30 minutes before the sample was applied. The resolution with the Flynn – de Mayo apparatus was better if the sample was applied 2.0 cm to the anode side of the peak rather than at the apex; this procedure was adopted routinely. With other types of apparatus, the sample was applied as close to the cathode as was convenient. Usually 10 μl of solution containing 10 to 100 μg of polysaccharide was used. A suitable direct-current potential was employed for sufficient time to produce adequate migration; in the horizontal apparatus, 70 volts for 16 hours was found to be suitable. Upon completion of the run, the tank was opened, the rack of papers was removed, the wet ends were cut off with scissors, and the paper was hung in an oven circulating air at 90° C to dry.

Staining

Method A.—The strips were stained for 1 minute in 0.5% (w/v) methylene

blue in 50% (v/v) ethanol, washed for 5 minutes each in two changes of 1% (v/v) acetic acid in 50% (v/v) ethanol and two changes of 50% (v/v) ethanol, then dried at 90° C. The acid mucopolysaccharides appeared as blue spots on a pale-blue background.

Method B.—A 1.0% (w/v) solution of Alcian blue 8GS in 95% ethanol was diluted, just prior to use, with nine parts of 0.1 *M* citrate buffer, pH 3.0, in 50% (v/v) ethanol. The strips were stained in this solution for 4 hours at room temperature with occasional agitation, then differentiated for three 15-minute periods in the buffered ethanol at 40° to 50° C, and dried in the 90° C oven for 30 minutes. The acid mucopolysaccharides appeared as dark-blue spots against a pale-blue background. Somewhat better staining of hyaluronate, although with darker background, is achieved by staining for 16 hours.

Electroosmosis

The electroosmotic migration of the medium was measured by application of 6% (w/v) dextran in isotonic saline (Intradex, Glaxo-Allenburys) to the paper. After completion of the run, the paper was dried, stained by spraying with 95% (v/v) ethanol containing 1% (w/v) resorcinol, 5% (w/v) trichloroacetic acid, and 0.5 *N* hydrochloric acid, and developed at 100° C for 10 minutes. Dextran appeared as a dark-brown spot on a pale background. This stain was superior to that suggested by Kunkel and Tiselius (39).

Calculation of Distance Migrated

The appearance of the mucopolysaccharide after migration and staining was usually slightly elliptical with the long axis in the direction of movement and a tail of diminishing intensity extending toward the origin (e.g. Fig. 2). For this reason, the distance migrated was calculated as the furthest distance of the stain from the point of application less one-half the width of the spot, the latter measured perpendicular to the direction of motion. A correction for electroosmosis was applied to each value.

Experimental

Staining

Both the methylene blue and Alcian blue stains described above were developed by systematic study of such variables as duration of staining, concentration of dye, composition of the solvent, composition of the washing medium, and time and number of washes. The conditions recommended are those which gave the maximal contrast between background and polysaccharide. Other basic dyes such as methyl violet, Janus green B, and basic fuchsin could be substituted for methylene blue with little loss in sensitivity or contrast. A number of other stains and modifications thereof were studied in some detail. For a variety of reasons, the dyes recommended are thought to be superior for general use. The sensitivities of several common staining procedures were compared (see Table I). The colloidal iron stain (31) gave deeply staining

TABLE I

Sensitivity of stains for acid mucopolysaccharides on filter paper

Stains and references	Lowest detectable weight (μg)	
	Hyaluronate	Chondroitin-4-sulphate
Colloidal iron		
(Feeney and McEwen (31))	1	10
Toluidine blue		
(Kerby (27))	5	0.1
(Hamerman (29))	10	0.1
Methylene blue		
(This paper)	0.1	0.1
Alcian blue		
(Feeney and McEwen (31))	0.2	0.2
(This paper) 4 hours	0.2	0.05
16 hours	0.05	0.02

NOTE: Ten microliters of water containing differing amounts of the polysaccharide was applied to Whatman No. 1 paper, dried with a hair dryer, and stained. The end point was the lowest concentration on the lower surface with central staining clearly deeper than the background. (The tendency to stain deeply at the periphery of the spot was neglected in this comparison.)

backgrounds against which the polysaccharides were difficult to visualize. The other stains differed mainly in sensitivity. The superiorities of methylene blue and Alcian blue in this respect were marked, especially if hyaluronate be considered. In our hands, methylene blue has not been uniformly successful with undegraded hyaluronate. For this reason, we are presently employing Alcian blue as a routine method.

Electrophoresis

Helmholtz (40) and Smoluchowski (41) demonstrated that the distance migrated by a charged particle is proportional to the potential gradient in the paper and to the time during which the field is applied. The data of Fig. 1 indicate that the migration of acid mucopolysaccharides follows the Helmholtz–Smoluchowski law for times up to 16 hours and for potential gradients up to 3 volts per cm. Within these limitations, the migration of the polysaccharide may be regarded as truly 'electrophoretic' in nature, that is, free of artifacts introduced by non-uniform potential gradients and varying composition of the electrolyte in the paper. A mobility (u') may be calculated from the equation $d = u'.V/l.t$ where d = distance migrated, V/l = voltage gradient *in the paper*, and t = duration of the run. However, this mobility (u') may differ from the mobility (u) in free electrophoresis, even for the same medium. Possible reasons for this will be discussed below.

Factors Affecting Mobility

The composition of the supporting medium affects electrophoretic migration markedly because of its effect on the charge density of the migrating particle and because of its relation to the conductivity of the supporting medium. In zone electrophoresis, the effect of the electrolyte on the surface charge and

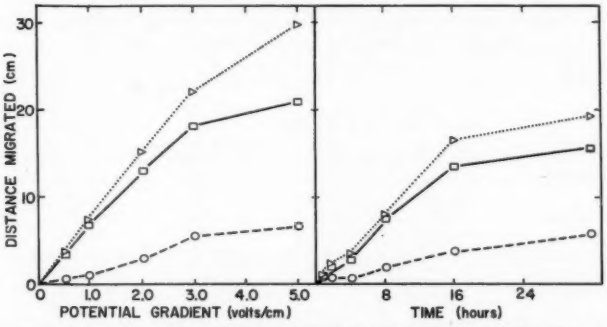

FIG. 1. The conformity of electrophoretic migration to the Helmholtz–Schmoluchowski law. Ten microliters of a 1.0% (w/v) solution of degraded hyaluronate (○), chondroitin-4-sulphate (□), and heparin (▷) was applied in duplicate to Munktell No. 20 (150 g) paper in the Flynn – de Mayo apparatus and allowed to migrate in lithium acetate, ionic strength 0.10, for 16 hours under potential gradients (in the paper) of 0.5, 1.0, 2.0, 3.0, and 5.0 volts per cm and for 1, 2, 4, 8, 16, and 32 hours under a potential gradient of 1.67 volts per cm. The strips were stained with methylene blue. A single separate experiment was performed for each different combination of the two variables.

absorptive properties of the supporting medium should also be considered in view of their relation to electroosmosis and tailing phenomena. An empirical study of a variety of common electrolytes indicated that lithium salts offered best resolution of hyaluronate, chondroitin sulphate, and heparin. The buffering qualities of the medium seemed unimportant so long as conditions were maintained which assured complete ionization of the carboxyl and ester sulphate groups. As shown in Table II, the mobility of the polysaccharides increased as

TABLE II

The effect of the ionic strength of the electrolyte on mobility

Ionic strength	Distance (cm) migrated by: Hyaluronate	Chondroitin-4-sulphate	Heparin
0.05	6.38	15.77	18.09
	6.39	15.79	18.64
0.10	5.50	15.14	16.48
	5.56	15.23	16.55
0.50	1.31	5.97	8.19
	1.32	6.00	8.52

NOTE: Ten microliters, containing 100 μg of the polysaccharide was applied to Munktell No. 20 (150 g) paper. The experiment was performed in the Flynn – de Mayo apparatus applying 2.0 volts per cm for 16 hours in a lithium acetate solution of the stated concentration. The strips were stained with methylene blue.

the ionic strength of the medium was reduced. An ionic strength of 0.10 was chosen because further reduction in concentration seemed likely to lead to pronounced effects from the sample electrolytes and further increase to marked reductions in the distance of migration. The data of Table III illustrate the superior separation of chondroitin-4-sulphate from heparin when lithium rather

TABLE III

The effect of the ions of the electrolyte on mobility

Electrolyte	Mobility of:		Distance (cm) migrated by:		
	Anion	Cation	Hyaluronate	Chondroitin-4-sulphate	Heparin
Lithium acetate	Slow	Slow	2.63	12.95	14.74
			2.71	13.02	15.20
Lithium sulphate	Fast	Slow	9.62	20.82	23.82
			9.66	21.05	24.64
Potassium acetate	Slow	Fast	5.10	14.20	15.80
			5.61	14.27	16.00
Potassium sulphate	Fast	Fast	8.88	18.79	20.22
			8.89	18.92	20.65

NOTE: The conditions were as described below Table II, using electrolytes of ionic strength 0.10.

than potassium salts were employed. Mobility appeared to be increased by the presence of a rapidly migrating anion, as shown by a comparison of the results with acetate and sulphate, but the data available do not constitute a rigorous test of this hypothesis. In general, we have employed lithium sulphate as a supporting electrolyte when the separation of acid mucopolysaccharides has been the principal object of the experiment and lithium acetate – acetic acid buffer when, in addition, control of the pH of the medium has seemed desirable.

The small but significant effect of the width of the strip on mobility is shown in Table IV. Some data were obtained suggesting that the results were more reproducible if a single wide sheet rather than separate strips was employed.

TABLE IV

The effect of strip width on mobility

Width of strip (cm)	Distance (cm) migrated by:		
	Hyaluronate	Chondroitin-4-sulphate	Heparin
2.0	5.12	13.52	17.97
	5.26	13.62	17.97
3.0	4.99	13.19	17.78
	5.06	13.27	17.86
4.0	4.74	12.90	17.74
	4.74	12.22	17.79
5.0	4.27	12.23	17.71
	4.81	11.33	17.81
6.0	4.34	11.14	17.51
	4.53	11.53	17.55

NOTE: The conditions were as described below Table II using lithium acetate, ionic strength 0.10, as electrolyte.

However, uniform staining of large single sheets is difficult; therefore separate strips for each sample have been used for most of our work. The data of Table IV demonstrate the need for consistent strip widths if critical comparisons are to be made.

During our early experiments, a daily increase in the relative mobility of hyaluronate was noted in association with a loss in the viscosity of the solution.

The effect on mobility of depolymerization of the polysaccharides under mild conditions is illustrated in Table V. The effect was pronounced for hyaluronate, the only one of the three polysaccharides which exhibits appreciable viscosity

TABLE V

The effect of alkaline degradation on mobility

Polysaccharide	Distance (cm) migrated by:		
	Hyaluronate	Chondroitin-4-sulphate	Heparin
Undegraded	1.03	14.05	17.94
Degraded	2.73	13.99	17.12

NOTE: One-half gram of the polysaccharide was dissolved in 100 ml *N*/10 sodium hydroxide and allowed to stand for 24 hours at room temperature. After precipitation with 10 volumes of 95% (v/v) ethanol (containing 5% (w/v) sodium acetate and 5% (v/v) acetic acid) and washing, the degraded and untreated materials were subjected to electrophoresis as described under Table IV, but at 1.67 volts per cm for 22.5 hours.

in solution. For this reason, polysaccharides degraded in the manner described under Table V were used in all experiments where the effects on mobility of various experimental parameters were being studied. The effect of viscosity on mobility is illustrated further by the data of Table VI. An increase in

TABLE VI

The effect of concentration and volume of the applied solution on mobility

Weight of polysaccharide (μg)	Volume (μl)	Distance (cm) migrated by:		
		Hyaluronate	Chondroitin-4-sulphate	Heparin
20	5	6.11	15.28	17.88
		6.32	15.74	17.90
20	10	6.57	15.57	18.23
		6.72	15.74	18.70
20	20	7.69	15.67	18.83
		7.77	15.67	18.97
20	50	8.41	15.41	19.21
		8.97	15.90	19.26
25	10	8.42	12.30	18.29
		8.48	12.57	18.44
50	10	7.36	12.77	17.75
		7.42	12.97	17.77
75	10	6.13	12.16	17.49
		6.60	12.18	17.49
100	10	5.84	12.34	16.68
		5.90	12.47	16.69

NOTE: Except for the concentrations and volumes of the solutions applied to the paper, the conditions were as described below Table IV.

viscosity was associated with a decrease in mobility when either a constant weight of polysaccharide or a constant volume of solution was applied. Again the effect was pronounced only in the case of hyaluronate. A similar phenomenon has been noted in free electrophoresis (42).

Good results have been obtained with Munktell No. 20, Whatman No. 1, and Whatman No. 3MM papers. However, the thickness of the latter paper does lead to a deep background stain.

Applications

Paper

The principal use of this method in our laboratory has been for preliminary identification of the cutaneous acid mucopolysaccharides. The separation of 'degraded' samples of mucopolysaccharides by our original procedure is shown in Fig. 2. The separation of hyaluronate, chondroitin-4-sulphate, and heparin was clearly demonstrated, as well as the amount of electroosmotic movement in the paper. The heterogeneity of the commercial heparin was apparent.

The preliminary identification of the acid mucopolysaccharide from a fraction of rat skin is illustrated in Fig. 3. In this experiment, results with our current apparatus and staining methods are demonstrated. The streaking of the hyaluronate and the broad band for the unknown and the chondroitin-4-sulphate were not usually seen; these may be the result of the relatively large volume of solution applied. The sensitivity of the Alcian blue stain is well illustrated. Routinely, we use 10 μg each of hyaluronate and chondroitin-4-sulphate as reference standards. The intensity of the stain suggests that considerably smaller quantities would be detectable. However, the weight of polysaccharide which must be applied to the paper to produce a clear spot after electrophoretic migration is somewhat greater than that suggested by direct staining (see Table I).

The homogeneity of a polysaccharide may be assessed objectively by analysis of strips cut from the filter paper. In our experience, this approach was more reliable than photometric scanning of the strip or elution and estimation of the dye. Such an experiment is illustrated in Fig. 4 for which the same polysaccharides and conditions were employed as in Fig. 2. The common finding of material remaining at the point of application was illustrated by all the curves and was especially evident in the cases of hyaluronate and chondroitin-4-sulphate. This artifact can be minimized by vigorous centrifugation of the solution before application. The single, sharp band characteristic of a homogeneous polysaccharide is illustrated by the pattern for the hyaluronate. The chondroitin-4-sulphate was contaminated with a trace impurity with the mobility of hyaluronate. The heparin was obviously heterogeneous. The superiority of analysis over staining as a method of recognizing heterogeneity is evident by comparison of Figs. 2 and 4.

Other Types of Electrophoresis

The conditions developed for paper electrophoresis were found to be directly applicable to other types of zone electrophoresis. The separation of two polysaccharides by electrophoresis on sponge rubber is illustrated in Fig. 5. The separation of the two polysaccharides was good. Rubber sponge could probably

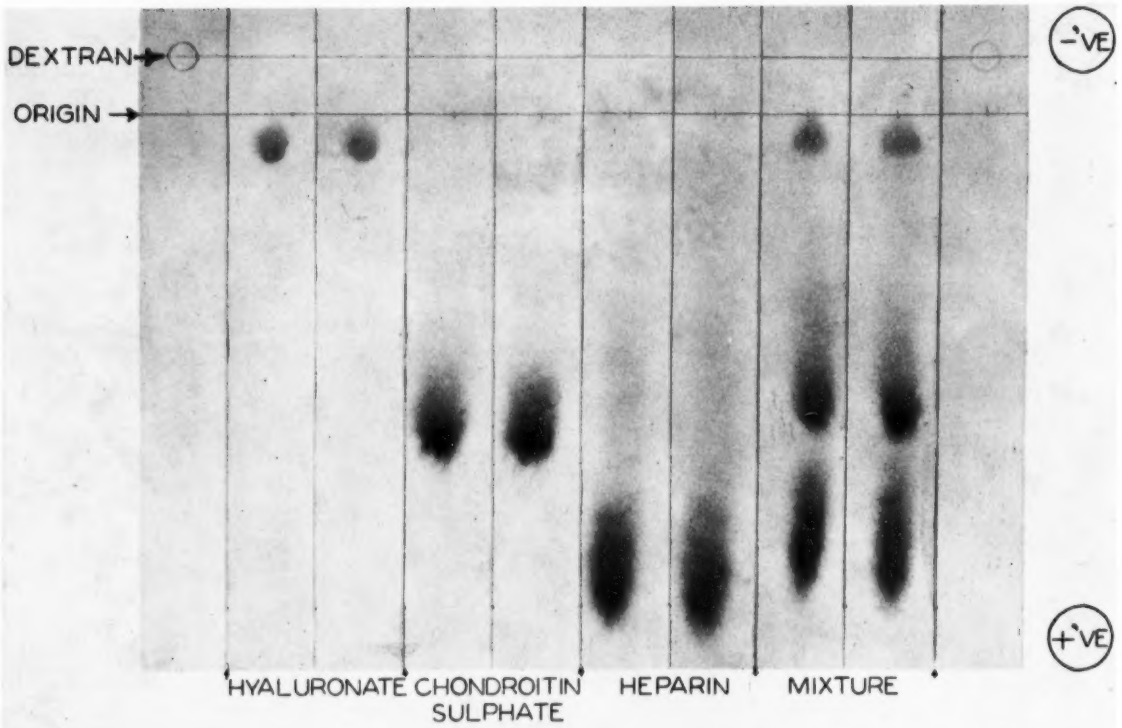

FIG. 2. Typical results. The separation of 100 μg of degraded hyaluronate, chondroitin-4-sulphate, and heparin (see Table V) applied in 10 μl of water to Munktell No. 20 (150 g) paper in the Flynn–de Mayo apparatus and using as electrolyte lithium sulphate, ionic strength 0.10, and a potential gradient of 3.0 volts per cm applied for 11 hours. The strips were stained with methylene blue.

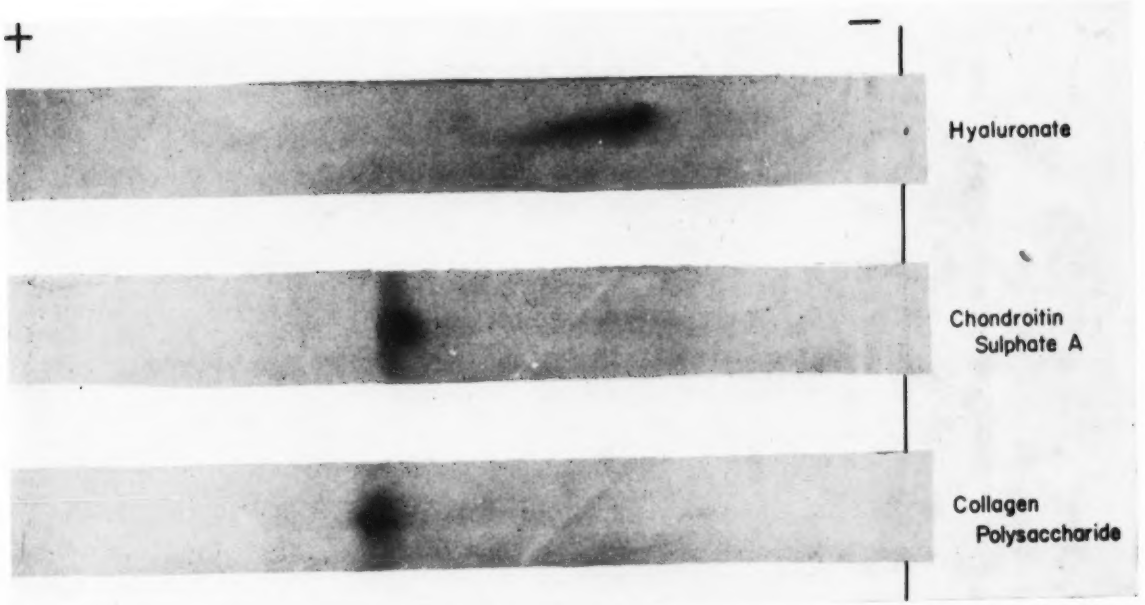

FIG. 3. Separation of polysaccharides of cutaneous collagen. Twenty microliters of solution containing 20 μg of purified polysaccharide was applied to Whatman No. 1 paper in the horizontal apparatus and 70 volts was applied for 16 hours, using lithium acetate, pH 3.5 and ionic strength 0.10. The paper was stained with Alcian blue.

be used in the place of celite for the separation of cutaneous acid mucopolysaccharides (46).

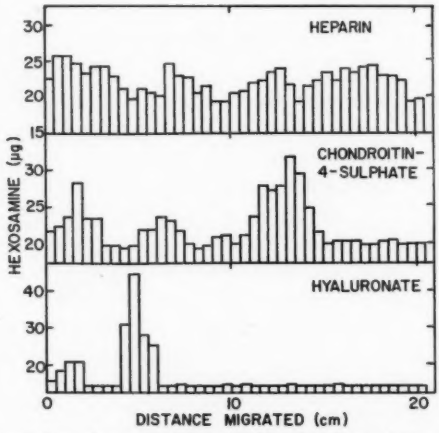

FIG. 4. The separation of mucopolysaccharides by filter paper electrophoresis with analysis of strips. Twenty microliters of solution containing 200 μg of polysaccharide was applied to Munktell No. 20 (150 g) paper. Two volts per cm was applied for 16 hours, using as electrolyte lithium acetate, ionic strength 0.10. The strips were dried, cut transversely into 5 mm bands, each of which was hydrolyzed and analyzed for hexosamine (43).

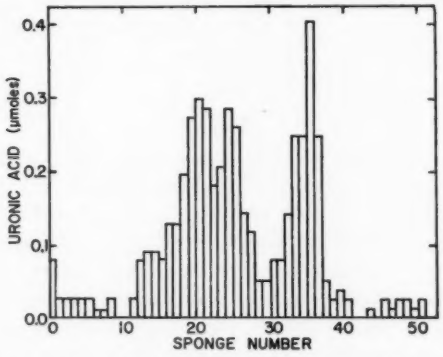

FIG. 5. Separation of polysaccharide mixtures on sponge rubber. Using the apparatus of Mitchell and Herzenberg (44) with lithium sulphate of ionic strength 0.10, sponge No. 3 was saturated with 3.6 ml of a solution containing 0.05% (w/v) of both hyaluronate and chondroitin-4-sulphate; 75 volts was applied to the sponges for 16 hours. The fluids were expressed from each sponge and analyzed for uronic acid (45).

Similar separations can be achieved by the use of continuous flow ('curtain') electrophoresis as illustrated in Fig. 6. Evidence for the presence of three acid mucopolysaccharides in a fraction of rat skin was obtained by examination of the stained sheet. Analysis of the collected fluid gave somewhat less convincing evidence of distinct fractions.

These applications serve to indicate the general applicability of the procedure to problems of separation of the acid mucopolysaccharides.

Discussion

Staining

Practically all the published histochemical stains for acid mucopolysaccharides have been used to locate these compounds on filter paper but two important requirements have frequently been overlooked. Firstly, the solvent for the dye should be one in which the mucopolysaccharides are insoluble. Even though the dye may form an insoluble complex with the polysaccharide, if such a solvent be not used the polysaccharide dissolves quickly, and, in our experience, the deposition of dye in the paper is slight. Therefore, ethanol was incorporated into the solvents for the stain and rinse. The reason for this requirement may be the absence of the occluding action of the denatured tissue proteins, such as is encountered in fixed tissues. Secondly, the presence of free carboxyl and other anionic groups in cellulose produces a tendency for objectionable background staining (47). Several of the published staining procedures do not recommend washing the stained strip to reduce the background stain; as a result, much of the sensitivity is lost. A suitable washing procedure is essential if the optimal sensitivity is to be achieved.

Methylene blue was chosen as a stain because the contrast seemed more striking than with the other basic dyes tested. However, the greyish shade of methylene blue is less striking than the blue green of the Alcian blue. Alcian blue has been adopted by us as a routine stain because of its improved visibility and greater reliability (described above).

Electrophoresis

Lithium acetate (without added acetic acid) was investigated initially because the low mobility of its ions reduced the conductivity of the solution and, hence, minimized the production of heat in the paper. The reason for the superior separation of the acid mucopolysaccharides achieved with this electrolyte is not apparent. The incorporation of a buffer into the medium seemed unnecessary since the mucopolysaccharides are strong electrolytes with a pK for the carboxyl group close to 4 (48); thus, the pH should not affect ionization appreciably at values more alkaline than 5.5. The change in pH due to electrolysis was found to be insignificant in the subcompartments into which the paper was dipped.

FIG. 6. Separation of cutaneous polysaccharides by continuous flow electrophoresis. The Spinco model CP continuous flow electrophoresis apparatus was used with lithium acetate, pH 3.5 and ionic strength 0.10, as the electrolyte. The wick capillaries were both adjusted to 9.4 cm to balance the background flow; the overflow tube was adjusted to 4.0 cm. A potential of 80 volts was applied, giving a current of 19.3 ma. A 1.0% (w/v) solution of a fraction of the cutaneous polysaccharides was applied at about 0.2 ml per hour (10-mm diameter tube, Duodial setting 6.25). After 16 hours, the run was stopped, the curtain was removed, dried, and stained with Alcian blue, and the tubes were analyzed for uronic acid (45).

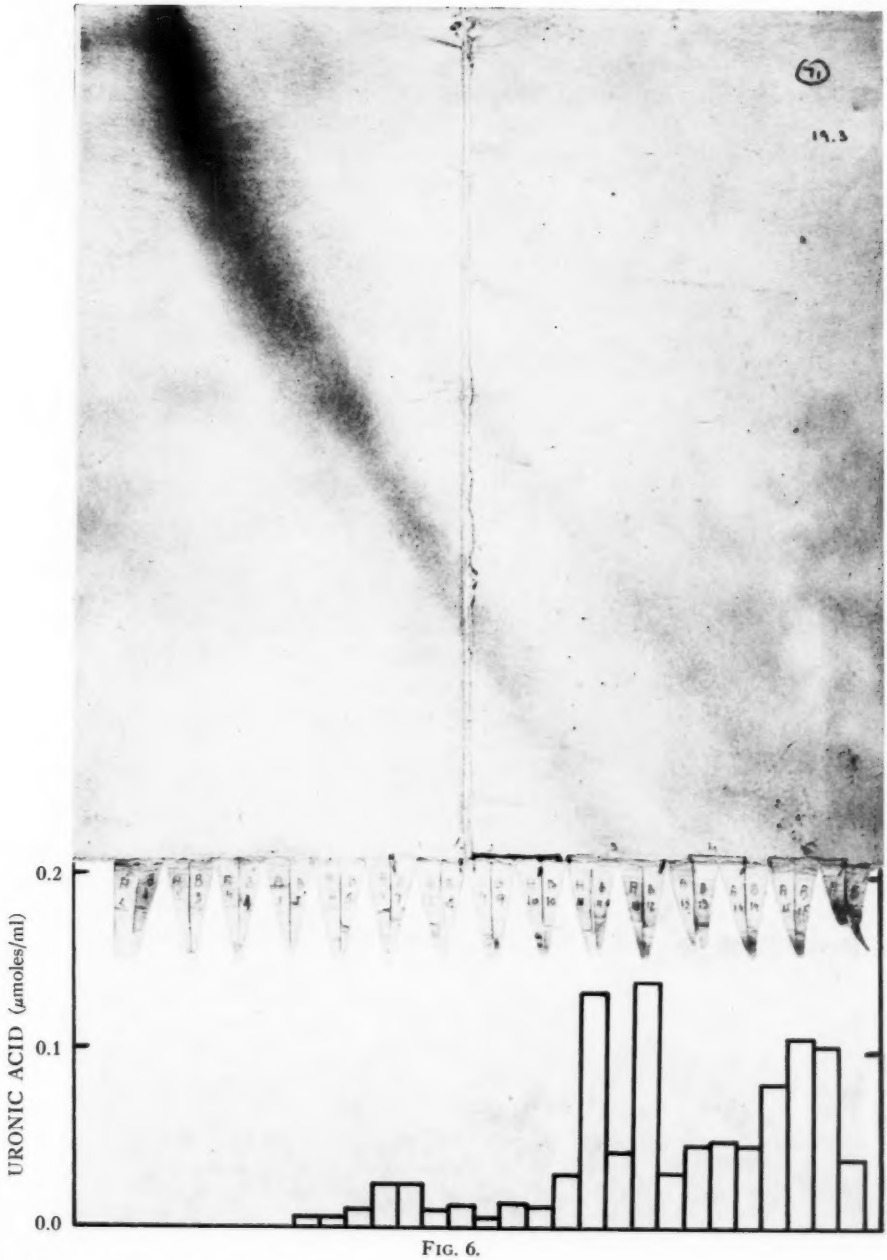

FIG. 6.

Mobility

By utilizing the Kunkel and Tiselius correction (39) for the tortuosity of the path of migration in filter paper, the mobility in free solution may be calculated. The conductivity of the buffer and the dimensions of the moistened paper were determined to obtain the necessary data. The values calculated in this manner fall as close to the values reported for free electrophoresis as might be expected considering the differences in electrolyte (see Table VII).

TABLE VII

The mobilities of the acid mucopolysaccharides

Polysaccharide	Mobility (cm^2/volt-sec$\times 10^4$)		
	Paper	Free	Reference
Hyaluronate	0.57 ± 0.19	0.9–1.1	49
		1.2	50
Chondroitin-4-sulphate	1.89 ± 0.35	1.7	50
Heparin	2.23 ± 0.40	0.8–2.4	51
		1.5	52

NOTE: The conditions for the paper electrophoresis were as described under Fig. 2. The values given are the mean and standard deviations from five independent duplicate runs. The values for free electrophoresis were obtained from the literature (see reference).

Application

Good separations have been achieved by application of our general conditions to a wide variety of apparatus. In general, we have used lithium sulphate or lithium acetate – acetic acid with ionic strength 0.10, a potential gradient of 2.0 to 3.0 volts per cm in the supporting medium, and a run of sufficient duration to allow heparin to migrate close to the end of the support. Samples have usually been applied as close to the cathode as is convenient. Under these conditions, results have been uniformly successful.

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MECHANISM OF SELECTIVE BLOCKADE OF CYCLOPROPANE-ADRENALINE CARDIAC ARRHYTHMIAS BY DICHLOROISOPROTERENOL[1]

MORLEY C. SUTTER[2] AND PETER E. DRESEL

Abstract

We reported previously that in cyclopropane-anesthetized dogs dichloroisoproterenol (DCI) sometimes fails to block adrenaline-induced bigeminal cardiac rhythm although it always prevents ventricular fibrillation. The present experiments demonstrated that in animals in which a large dose of DCI did not increase the adrenaline threshold for bigeminy, previously ineffective mechanical elevation of the blood pressure induced bigeminy. Neither methoxamine nor DCI alone caused bigeminal rhythm, but their combination effectively induced bigeminy of long duration. These results are interpreted to indicate that apparent failure of DCI to block adrenaline-induced bigeminy is not due to inability to antagonize this cardiac action, but is due to a combination of the intrinsic cardiac sympathomimetic activity of DCI with an increase in blood pressure induced by the subsequent injection of adrenaline.

We have reported recently that small doses of adrenaline injected intravenously into cyclopropane-anesthetized dogs can induce a constantly coupled bigeminal rhythm. We showed that this arrhythmia could not be due to the induction of a focus of automaticity in the ventricle and that the presence of a sympathomimetic amine with cardiac actions together with an increase in blood pressure was required to produce it (1). One of the characteristics distinguishing this arrhythmia from ventricular fibrillation induced by larger doses of adrenaline was the effect of dichloroisoproterenol (DCI). This agent, which blocks cardiac actions of adrenaline (2, 3), appeared to be much more effective in preventing ventricular fibrillation induced by adrenaline than in raising the threshold dose of adrenaline for the induction of the bigeminal rhythm. The present experiments were designed to investigate the mechanism of the failure of DCI to block consistently the bigeminal rhythm induced by minimal doses of adrenaline.

Methods

The methods used in these experiments were identical with those described in our previous communication (1). After thiopental induction, 20% cyclopropane in oxygen was administered by means of a Palmer pump at a rate of 18 strokes/minute with a tidal volume adjusted to provide adequate expansion of the lungs. All animals were vagotomized. Carotid artery pressure and Lead II electrocardiograms were recorded on a Grass polygraph. Systemic arterial pressure was altered by temporary occlusion of the thoracic aorta or by the

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Contribution from the Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, aided by grants from the National Research Council of Canada.
[2]Medical Research Fellow, National Research Council of Canada.

use of a pressure regulator connected to the aorta below the renal arteries. The regulator consisted of a reservoir connected to a large air pressure tank and was primed with fresh dog blood which, in some cases, was diluted by not more than 50% with 6% dextran in saline. Adrenaline was injected intravenously during a 60-second period in doses of 0.1 to 2.0 μg/kg or was infused at rates varying from 0.025 to 2.0 μg/kg/minute. Methoxamine (0.2 or 0.4 mg/kg) was injected intravenously in 1 minute. Dichloroisoproterenol was given intravenously in a dose of 4.0 mg/kg over a 5-minute period, and 30 minutes was allowed for the development of maximal blockade before subsequent tests.

Results

The threshold dose of adrenaline required to induce bigeminy was determined in 13 dogs before and after treatment with 4.0 mg/kg of DCI. This blocking agent increased the threshold in six animals, had no effect in four, and decreased the threshold in the remaining three dogs. These experiments confirm our previously reported results (1).

The effect of mechanical elevation of the systemic blood pressure, before and after DCI, was studied in 7 of these 13 animals. No effect was seen before DCI in any of the experiments. After DCI, occlusion of the aorta or infusion of donor blood to increase the systemic pressure induced bigeminy in three of the seven animals.

There appeared to be a clear relation between the effect of DCI on the threshold dose of adrenaline and the electrocardiographic response to subsequent mechanically induced increases in systemic pressure. The three animals which showed bigeminy in response to an increased blood pressure after DCI were those in which no elevation of the adrenaline threshold was effected by the blocking agent. The remaining four animals which did not show bigeminy in response to an increased blood pressure were those in which the adrenaline threshold was elevated by the blocking agent.

Figure 1 shows the response to occlusion of the thoracic aorta in an animal in which DCI failed to increase the adrenaline threshold. Soon after aortic occlusion, a pulse deficit unaccompanied by a change in the electrocardiogram was observed. This is probably analogous to the pulsus alternans described in detail by Ellis (4). The configuration of the electrocardiogram then changed suddenly to that of a typical bigeminal rhythm indistinguishable from that produced by adrenaline. The sinus tachycardia and decreased height of the QRS complex seen in this record are a common response to DCI. There was no clear-cut relation between the degree of the sinus tachycardia induced by DCI and subsequent failure to block bigeminy.

The combined effect of methoxamine, a sympathomimetic pressor amine lacking cardiac actions, and of DCI was examined in a series of eight dogs. Four of these animals received methoxamine followed 5 minutes later by DCI, while the others received DCI followed 30 minutes later by methoxamine. Neither drug alone produced bigeminy in these animals. Combination of the

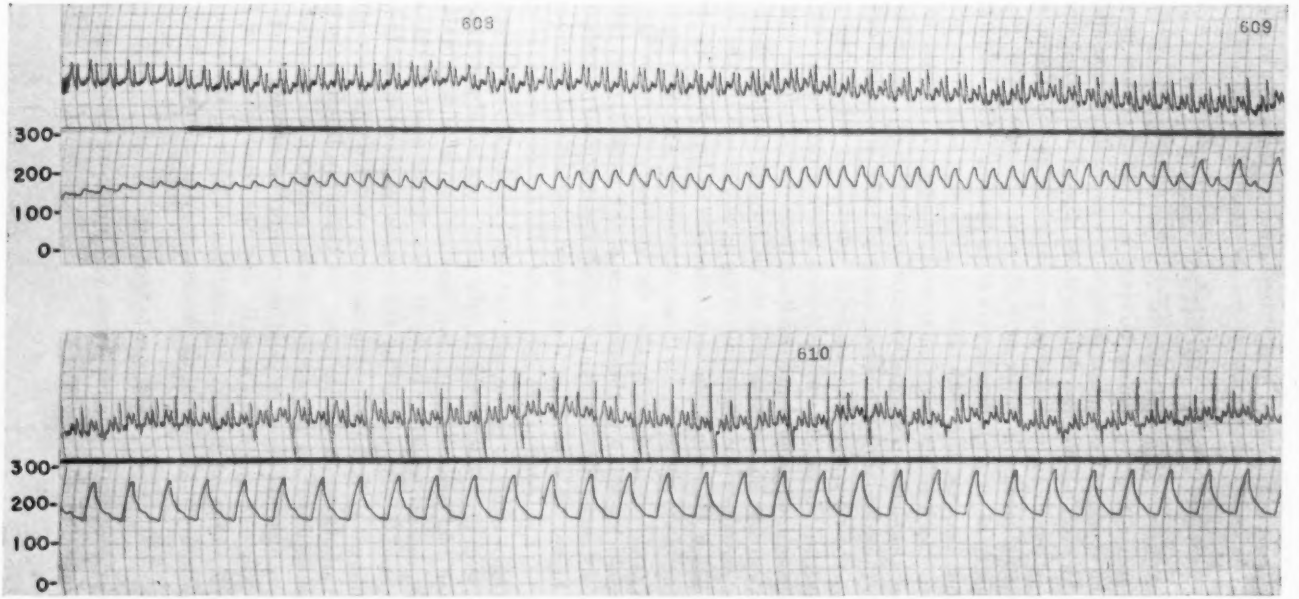

FIG. 1. Continuous record of Lead II electrocardiogram and arterial pressure on occlusion of the thoracic aorta (at signal) 40 minutes after administration of 4.0 mg/kg of DCI. Pulse deficit occurs 14 seconds, bigeminy 21 seconds, after start of occlusion.

two agents resulted in bigeminy of up to 15 minutes' duration in seven of the eight animals.

Discussion

Our previous observations (1) indicated that two factors, a sympathomimetic amine with cardiac actions and an increase in systemic blood pressure, are required for the consistent induction of bigeminy in cyclopropane-anesthetized dogs. The failure of DCI to raise the threshold dose of adrenaline required for the induction of bigeminy in half of our preparations may be due either to the high but variable cardiac intrinsic activity reported previously (2, 3) or to variable failure to block a cardiac action of adrenaline. The latter possibility appears remote because the high dose of DCI employed has been shown consistently to block other ventricular arrhythmias (1, 3) and to decrease greatly cardiac responses to sympathomimetic amines (2). We believe that in dogs in which the adrenaline threshold appeared to be unchanged or decreased subsequent to the administration of DCI, adrenaline was acting only as a peripheral vasoconstrictor, its cardiac action being greatly attenuated. However, the increase in blood pressure resulting from the peripheral vasoconstriction plus the cardiac stimulation due to DCI itself fulfilled the conditions known to be necessary for bigeminy. This is suggested by the relation of apparent failure of DCI blockade with induction of bigeminy by mechanical elevation of the blood pressure. In those experiments in which an increase in threshold did occur, it is probable that the intrinsic activity of DCI was insufficient to yield bigeminy in the presence of the hypertension produced by either the control threshold dose of adrenaline or mechanical procedures. Doses of adrenaline higher than the original threshold dose induced bigeminy in these animals. We have no evidence to determine whether this response was due to the greater pressor effect or to the fact that blockade by DCI is competitive, or both. The dose of DCI used in the experiments reported above was chosen to yield maximal blockade without the production of direct quinidine-like myocardial depression, which may result from higher doses of the agent (2).

Our interpretation is supported by the results obtained when the action of DCI was combined with that of methoxamine. Methoxamine is a vasopressor agent without adrenergic cardiac actions. DCI does not block vasoconstriction, and again the intrinsic cardiac action of DCI appears to combine with the pressor effect of methoxamine to yield the conditions necessary for induction of bigeminy. We are unable to explain why the combination of DCI with methoxamine is more effective than its combination with adrenaline in inducing bigeminal rhythm. An additional mechanism may be involved.

Acknowledgment

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NOTES

SULPHYDRYL-INITIATED AGGREGATION OF MERCAPTALBUMIN IN 0.1 *N* SODIUM CHLORIDE SOLUTIONS

W. Bushuk

In studies of mercaptalbumin (monomer, twice crystallized, Nutritional Biochemicals) used as a model substance in investigations of the sulphydryl groups of flour proteins, it was observed by light scattering that the slow molecular aggregation which occurs in solutions of low concentration can be prevented by N-ethylmaleimide (NEMI) or by iodate. Though detailed study of this observation is not contemplated, it is reported since it may be useful to others.

Figure 1 gives the transverse scattering relative to 60 for pure benzene, for solutions of 0.125% mercaptalbumin in 0.1 *N* sodium chloride and in 0.1 *N* sodium chloride containing 3×10^{-4} *N* NEMI kept for 30 hours at room temperature (26° C). The intensity of scattered light was measured on a Wippler–Scheibling light-scattering photometer (1). Solutions were clarified by filtration

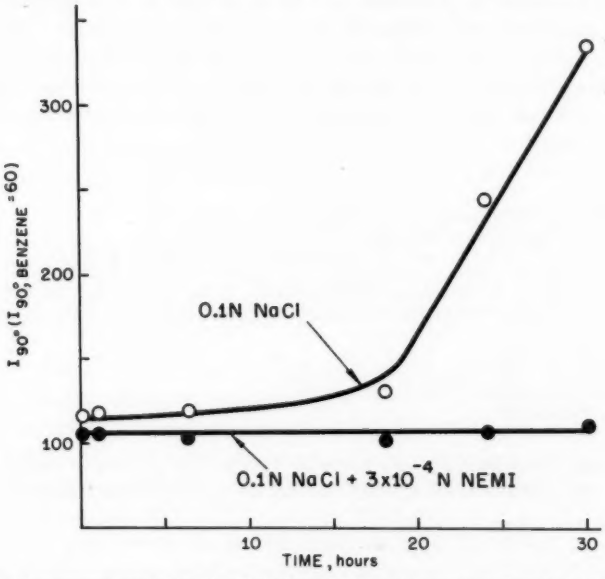

FIG. 1. Transverse scattering by 0.125% solutions of mercaptalbumin in 0.1 *N* NaCl and in 0.1 *N* NaCl containing 3×10^{-4} *N* N-ethylmaleimide (NEMI) kept at room temperature.

through an ultrafine sintered-glass filter followed by centrifugation for 1 hour at 30,000×gravity.

The scattering of mercaptalbumin in 0.1 *N* sodium chloride solutions increased quite markedly during the 30 hours of observation. On the other hand, the scattering by mercaptalbumin treated with NEMI to block the sulphydryl groups remained essentially constant. Amperometric titrations (2), which gave 1.1 sulphydryl groups per mole of albumin, showed that all of these sulphydryls were blocked by NEMI. Scattering by the iodate-treated mercaptalbumin solutions was initially slightly lower than that of the other two solutions; however, it also remained constant during subsequent storage.

The weight-average molecular weight of mercaptalbumin with the free sulphydryls was initially 106,000; this value increased to 400,000 after storage of the solutions at room temperature for 24 hours. The increase in molecular weight could not be reversed by dilution or by subsequent addition of NEMI, i.e. the aggregation seems to be irreversible. NEMI- and iodate-treated mercaptalbumin had molecular weights of 92,000 and 87,000 respectively. The magnitude of the initial molecular weight suggests that some aggregation occurred in 0.1 *N* sodium chloride solution during the time required to clarify the solutions and also that some aggregation had occurred in the original solid material (accepted molecular weight for mercaptalbumin is 67,000).

Results described in this note can be explained by a sulphydryl-initiated chain reaction with disulphide bonds with the formation of intermolecular disulphide bonds. The mechanism was first proposed by Huggins, Tapley, and Jensen to explain the formation of protein gels in urea solutions (3) and subsequently was found operative in the aggregation of bovine plasma albumin during denaturation by heat (4) and urea (5) and in a number of other types of protein denaturation (6).

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Received April 7, 1961.
Grain Research Laboratory,
Board of Grain Commissioners for Canada,
Winnipeg 2, Manitoba.

THE DISSIMILAR SELECTIVE TOXICITY OF TWO VINYL PHOSPHOROTHIONATE ISOMERS (THIONOPHOSDRIN)

E. Y. Spencer

The first toxic organophosphorus compounds developed were indiscriminate in their toxicity to mammals and insects. They were shown to be active

inhibitors of the cholinesterases. The introduction of malathion (O,O-dimethyl-S-(1,2-dicarbethoxyethyl)phosphorodithioate initiated a series with low mammalian toxicity yet still retaining good insecticidal activity. Of several explanations offered for this selective toxicity (1, 6) the most satisfactory was proposed by O'Brien (2) and subsequently confirmed by him and his colleagues (3). It is applicable where a thionosulphur group and a carboxy-ester group are an integral part of the organophosphorus compound. All organisms convert the phosphorothionate to the more toxic phosphate and, in addition, the non-susceptible organism detoxifies the phosphate by hydrolyzing the carboxy ester to the non-toxic free carboxy acid phosphate.

In the course of an investigation of the chemical properties (4) of the two geometrical isomers of a carboxy-ester vinyl phosphate, O,O-dimethyl 1-methyl-2-carboxyethyl vinyl phosphate (Phosdrin), and of their degradation in the pea plant (5) it was of interest to synthesize the corresponding thionophosphate isomers and determine whether they showed the same selective toxicity as found by O'Brien *et al.* (3) since the two essential components were present. Synthesis of the thionophosphate by reaction of dimethyl phosphorothionochloridate with the sodium salt of methyl acetoacetate yielded only the trans isomer (7). The cis isomer was obtained by ultraviolet irradiation of the trans isomer followed by separation of the equilibrium mixture on an alumina column. Comparative toxicities were established by topical application of an acetone solution to the female housefly and to the American roach and by intraperitoneal injection of an emulsion to the mouse. For comparative purposes the LD_{50} values for both geometrical isomers of the vinyl phosphates of Phosdrin and the vinyl thionophosphates of thionoPhosdrin are given.

Toxicity of Phosdrin and thionoPhosdrin isomers (LD_{50} mg/kg)

	Fly	Roach	Mouse	Toxicity ratio, mouse/fly
cis Phosdrin	0.27	4.7	2.0	7.4
cis thionoPhosdrin	3.2	17.0	17.0	5.3
trans Phosdrin	14.5	12.0	45.0	3.1
trans thionoPhosdrin	3.5	16.0	700.0	200.0

Although trans thionoPhosdrin is unusual in being more toxic to the housefly than the phosphate analogue, the relative toxicity of the others to the fly follows the normal pattern. Comparing the values for the mouse, the cis thiono isomer is the 'irregular' member of the series showing no selective toxicity by contrast with the low activity of the trans thiono isomer as predicted (3). The values for the American roach tend to fall between the unexpected ones for the housefly and the mouse. Apparently the mouse is able to tolerate a considerable amount of the trans thiono isomer due to its ability to detoxify the isomer. Presumably this is via a carboxy esterase as suggested by the potentiation of the activity with EPN, which inhibits carboxy esterases (9). The cis thiono isomer on the other hand appears to be unaffected by any detoxifying carboxy esterases and

is comparable in toxicity to the phosphate isomer. This is substantiated by the finding that no potentiation is shown with EPN (9).

It is not surprising that the two isomers differ in their relative toxicities since the reaction of the inhibitor with the specific enzyme can be greatly influenced by the shape of the R group esterified with the dimethyl or diethyl phosphate of many organophosphorus insecticides. This is shown particularly in the recent development of animal systemic insecticides (10). Further work is underway in an attempt to explain the difference in reactivity and selective toxicity of these thiono organo vinyl phosphate carboxy-ester isomers.

My thanks are due Mr. L. G. Crawford and Mr. J. Pawlik for carrying out the bio-assays.

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PESTICIDE RESERACH INSTITUTE,
CANADA AGRICULTURE,
UNIVERSITY SUB P.O. LONDON, ONTARIO.

NOTES TO CONTRIBUTORS

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Line drawings.—Drawings should be carefully made with India ink on white drawing paper, blue tracing linen, or co-ordinate paper ruled in blue only; any co-ordinate lines that are to appear in the reproduction should be ruled in black ink. Paper ruled in green, yellow, or red should not be used. All lines must be of sufficient thickness to reproduce well. Decimal points, periods, and stippled dots must be solid black circles large enough to be retained when reduced. Letters and numerals should be neatly made, preferably with a stencil (**do NOT use typewriting**), and be of such size that the smallest lettering will be not less than 1 mm high when the figure is reduced to a suitable size. Many drawings are made too large; originals should not be more than 2 or 3 times the size of the desired reproduction. Wherever possible two or more drawings should be grouped to reduce the number of cuts required. In such groups of drawings, or in large drawings, full use of the space available should be made; the ratio of height to width should conform to that of a journal page (4¾×7½ in.), but allowance must be made for the captions. **The original drawings and one set of clear copies (e.g. small photographs) are to be submitted.**

Photographs.—Prints should be made on glossy paper, with strong contrasts. They should be trimmed so that essential features only are shown and mounted carefully, with rubber cement, on white cardboard, with no space between those arranged in groups. In mounting, full use of the space available should be made. **Photographs are to be submitted in duplicate;** the duplicate set of grouped photographs should be unmounted.

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